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Pentamidine Is an Inhibitor of PRL Phosphatases with Anticancer Activity¹

Manas K. Pathak, Deepika Dhawan, Daniel J. Lindner, Ernest C. Borden, Carol Farver, and Taolin Yi²

Department of Cancer Biology, Lerner Research Institute [M. K. P., D. D., E. C. B., T. Y.], Taussig Cancer Center [D. J. L., E. C. B., T. Y.], and Department of Pathology [C. F.], The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Abstract

The PRL family oncogenic phosphatases are attractive targets for developing inhibitors as anticancer therapeutics given their potentially pathogenic role in human malignancies. Herein we demonstrate that pentamidine, an anti-protozoa drug with an unknown mechanism of action, is an inhibitor of PRLs with anticancer potential. Pentamidine at its therapeutic doses inhibited recombinant PRL phosphatases *in vitro* and inactivated ectopically expressed PRLs in NIH3T3 transfectants with an effective duration more than 24 h after a pulse cell treatment. The drug had *in vitro* growth-inhibitory activity against human cancer cell lines that express the endogenous PRLs. Pentamidine at a tolerable dose markedly inhibited the growth of WM9 human melanoma tumors in nude mice coincident with the induction of tumor cell necrosis and is capable of inactivating ectopically expressed PRL-2 in the cancer cells. These observations suggest the potential of pentamidine in anticancer therapies and may provide a basis for developing novel PTPase-targeted therapeutics.

Introduction

Protein tyrosine kinases and PTPases³ are critical intracellular signaling molecules and key targets for developing novel therapeutics (1). The potential of such targeted therapeutics has been well demonstrated by the successful treatment of human chronic myelogenous leukemia and gastrointestinal stromal tumors with the protein tyrosine kinase inhibitor STI-571 (2, 3), which targets bcr/abl or c-kit aberrantly activated in the malignancies. Given the critical role of

PTPases in intracellular signaling, inhibitors of the phosphatases might be expected to have therapeutic value. Thus far, few clinically useful inhibitors of PTPases have been reported, despite extensive efforts in the last decade to identify them (4).

The PRL family tyrosine phosphatases (PRL-1, PRL-2, and PRL-3) are highly attractive targets for developing inhibitors as novel anticancer therapeutics because overexpression of these phosphatases plays a potentially pathogenic role in human malignancies. *PRL-1* was identified more than 10 years ago as one of the genes expressed during liver regeneration (5). *PRL-2* and *PRL-3* were found more recently based their homology to *PRL-1* (6, 7). PRLs are closely related phosphatases with at least 75% amino acid sequence similarity (7). In normal adult tissues, PRLs are expressed predominantly in skeletal muscle with lower expression levels detectable in brain (*PRL-1*), liver (*PRL-2*), and heart (*PRL-3*; Refs. 5 and 7). Physiological functions of the PRLs are unclear at present, although the involvement of *PRL-1* in proliferation was suggested by its increased expression in regenerating liver (5). A potential role in the maintenance of differentiating epithelial tissues was proposed based on the selective expression of PRLs in terminally differentiated cells in kidney and lung (*PRL-1*; Ref. 8) as well as in mouse intestine (*PRL-3*; Ref. 9). Importantly, overexpression of *PRL-3*, resulting from gene amplification or other defects, was associated with tumor metastasis of human colorectal cancer (10, 11). The potential involvement of *PRL-3* overexpression in other human malignancies is indicated by the localization of the *PRL-3* gene at human chromosome 8q and by the observation that extra copies of this region are often found in advanced stages of different tumor types (10, 11). Consistent with an oncogenic role of PRL overexpression, ectopic expression of PRLs enhances cell growth, causes cell transformation, and/or promotes tumor growth in nude mice (5, 12). The oncogenic mechanism and regulated signaling events/molecules of the phosphatases remain undefined. Although PRLs could be inhibited by sodium orthovanadate (5, 13), which broadly inhibits all phosphatases (4), clinically useful and specific inhibitors of PRLs have not been reported.

Pentamidine [1,5-di(4-aminophenoxy)pentane] has been in clinical use for leishmaniasis, the hemolympathic stage of Gambian trypanosomiasis and *Pneumocystis carinii* pneumonia (PCP; Ref. 14), although its mechanism of action remains elusive. Trypanosomes actively transport pentamidine intracellularly, which might then interfere with DNA biosynthesis (14). However, the drug kills nonreplicating *P. carinii* and, thus, apparently functions independently of DNA biosynthesis (14). *In vitro* inhibition of group I intron splicing in *P. carinii* occurs at 250 μM (~150 $\mu\text{g}/\text{ml}$; Ref. 15), much higher than the therapeutic dosage of the drug (2–4 mg/kg; Ref. 16). Similarly, pentamidine inhibits constitutive brain

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² To whom requests for reprints should be addressed, at Department of Cancer Biology, Lerner Research Institute, NB4-67, The Cleveland Clinic Foundation, 9500 Euclid Avenue, NB4-67, Cleveland, OH 44195. Phone: (216) 445-9656; Fax: (216) 445-6269; E-mail: yit@ccf.org.

³ The abbreviations used are: PTPase, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase 1B; SSG, sodium stibogluconate; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; PRL, phosphatase of regenerating liver; IL, interleukin; GST, glutathione S-transferase; RT-PCR, reverse transcription-PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBMC, peripheral blood mononuclear cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

nitric oxide synthase only at a dose in the 100-1000 μM range (17, 18). The drug also has DNA-binding activity that appears unrelated to its pharmacological efficacy (19, 20).

Several lines of evidence suggest that the action of pentamidine against leishmaniasis, a tropical disease caused by a protozoan residing in host macrophages, might be mediated via host cellular targets and the host immune system. Pentamidine selectively targets intracellular leishmania in macrophages but not the free-living form of the protozoan (21) and has reduced anti-leishmania activity in immunodeficient mice in comparison with its action in immunocompetent hosts (22). In contrast, the anti-leishmania drug amphotericin B acts against both the intracellular and free-living forms of the protozoan (21) and is active in normal as well as in immunodeficient mice (22). The identities of potential host cellular targets of pentamidine and the mechanism of host immunity in the drug's anti-leishmania action have not been defined.

Interestingly, SSG had similar characteristics in its anti-leishmania action. SSG acted selectively against intracellular leishmania (21) and had severely impaired anti-leishmania activity in immunodeficient hosts (23), including mice lacking certain cytokines (*e.g.*, IFN, IL-4, and IL-12; Refs. 24–26). We demonstrated in a recent study (27) that SSG was a potent inhibitor of selective host cell PTPases, including SHP-1, which dephosphorylates Jak kinases to down-regulate cytokine signaling (28–33). Consistent with inhibition of SHP-1, SSG augmented cytokine-induced Jak2 phosphorylation and growth responses in hematopoietic cell lines (27). Given that a number of the cytokines (*e.g.*, IFNs and IL-12) signaling through the Jak/Stat pathway can activate macrophages to develop leishmanicidal activity (24, 26), SSG anti-leishmania activity might be mediated via inhibiting negative regulatory PTPases to augment the signaling and biological effects of cytokines. This putative mode of action provides a rational explanation for the selective activity of SSG against intracellular leishmania and its requirement for endogenous host cytokines. It is consistent with SSG enhancement of the anticancer effects of IFNs *in vitro* and in mouse models (34). Moreover, it suggests the possibility that pentamidine might function similarly as an inhibitor of PTPases and might have anticancer potential as a consequence.

In this report, we demonstrate for the first time that pentamidine is a potent inhibitor of selective PTPases with its PTPase specificity different from that of SSG. Pentamidine inhibits PTP1B and may potentiate cytokine signaling via this Jak PTPase (35). Interestingly, pentamidine inhibits the oncogenic PRL phosphatases and has growth-inhibitory activity against human cancer cell lines expressing PRLs and against WM9 human melanoma tumors in nude mice. As a clinically used drug with a novel mode of action, pentamidine may have potential for rapid incorporation into cancer therapies and might provide a basis for developing more effective and specific PTPase-targeted therapeutics.

Materials and Methods

Reagents. Pentamidine (Pentam 300, standard therapeutic grade) was from American Pharmaceutical Partners, Inc. SSG and GST fusion proteins of SHP-1, SHP-2, PTP1B, and

MKP1 have been described previously (27). cDNAs of human PRL-1, PRL-2 and PRL-3 coding region were derived by RT-PCR from H9 cells (36) and inserted in frame into the pGEX vector. GST fusion proteins of the PRL phosphatases were prepared from DH5 α bacteria transformed with the pGEX fusion protein constructs following established procedures (37). cDNAs encoding the PRLs tagged at the NH₂ terminus with the Flag epitope (38) were generated via recombinant DNA technique, sequenced to confirm their identities, and cloned into the pBabepuro (39) or pRK5 (30) vector. Anti-Flag monoclonal antibody (M2; Sigma), anti-phosphotyrosine monoclonal antibody (4G10; UBI), anti- β -actin monoclonal antibody (Pharmacia) and anti-SHP-2 polyclonal antibodies (Santa Cruz Technologies) were purchased from commercial sources. A synthetic phosphotyrosine peptide (R-R-L-I-E-D-A-E-pY-A-A-R-G; UBI) and DiFMUP (Molecular Probes) were purchased as substrates for PTPase assays.

In Vitro PTPase Assays and Immunocomplex PTPase Assays. *In vitro* PTPase assays were used to determine the effects of compounds on recombinant PTPases, following established procedures using a synthetic phosphotyrosine peptide (27) or DiFMUP (13) as the substrate. Briefly, individual PTPases (0.01 $\mu\text{g}/\text{reaction}$) in 50 μl of PTPase buffer [50 mM Tris (pH 7.4)] were incubated at 22°C for 10 min or as indicated in the absence or presence of inhibitory compounds. Substrates (0.2 mM phosphotyrosine peptide) were then added and allowed to react at 22°C for 18 h. PTPase activity of individual reactions was measured by adding 100 μl of malachite green solution (UBI) and then quantifying the amounts of free phosphate cleaved by the PTPase from the peptide substrate (27) by spectrometry ($A_{660\text{ nm}}$). Relative PTPase activities were calculated based on the formula [(PTPase activity in the presence of an inhibitory compound)/(PTPase activity in the absence of the compound) \times 100%]. Reactions performed under comparable conditions in the absence of recombinant PTPases only were used as controls and showed no detectable PTPase activity. PTPase assays using DiFMUP as a substrate were conducted following a previously described procedure (13). To assess the reversibility of PTPase inhibition, GST fusion proteins of the PTPases bound on glutathione beads (Pharmacia) were pre-incubated with cold Tris buffer [50 mM Tris (pH 7.0)] or Tris buffer containing the inhibitor at 4°C for 30 min. The beads were then washed three times in cold Tris buffer or not washed prior subjecting to *in vitro* PTPase assays.

Immunocomplex PTPase assays were performed to assess the effects of pentamidine on intracellular PTPases. Individual PTPases were immunoprecipitated from untreated or pentamidine-treated cells that were washed with fresh medium and then lysed in cold lysis buffer of PTPase assays [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, and 20 $\mu\text{g}/\text{ml}$ of Aprotinin]. The immunocomplexes were collected with protein G Sepharose beads (Pharmacia) and washed in cold lysis buffer for four times. Individual samples were then incubated in 50 μl of PTPase buffer [50 mM Tris (pH 7.4) and 0.2 mM phosphotyrosine peptide] at 22°C for 18 h. Malachite green solution (100 μl ; UBI) was added to each reaction, which was then

incubated at 22°C for 5 min before the measurement of $A_{660\text{ nm}}$ to quantify the amounts of free phosphate cleaved by the PTPases from the peptide substrate (27). Ten % of the contents of individual samples were also analyzed by SDS-PAGE/Western blotting to quantify the relative amounts of the phosphatase proteins. To assess the duration of pentamidine effects on the activities of intracellular PTPases, Flag-PRL-2-transfected cells were untreated or treated with pentamidine (1 $\mu\text{g/ml}$) for 5 min at 37°C, washed twice with culture medium to remove cell-free drug, and then incubated in fresh culture medium at 37°C for 24–72 h before termination by lysing the cells in cold lysis buffer of PTPase assays. Flag-PRL-2 was immunoprecipitated from the lysates and subjected to PTPase assays and SDS-PAGE/Western blotting as described above.

Cells, Cell Culture, Cell Growth Assays, and Transfection. NIH3T3 (40), WM9 (41), DU145 (42), C4–2 (43), Hey (44), SW480 (45), and A549 (46) cell lines have been described and were cultured in RPMI 1640 supplemented with 10% FCS. For the measurement of pentamidine effects on cell growth *in vitro*, cells were cultured in the absence (–) or presence (+) of various amounts of pentamidine for 6 days with viable cells quantified by MTT assays as described previously (27). Percentages of growth inhibition by pentamidine were calculated ($\pm x\%$)

The effects of pentamidine on intracellular PTPases were assessed using NIH3T3 or WM9 transfectants. NIH3T3 cells were transfected with the pBabepuro vector (V) or pBabepuro expression constructs of Flag-tagged PRLs using LipofectAMINE (Life Technologies, Inc.) following the manufacturer's procedures. Transfectants were selected in the presence of puromycin (0.5 $\mu\text{g/ml}$) for 2 weeks and expanded in culture without puromycin before their usage in measuring the effects of pentamidine on the PTPase activities of intracellular Flag-PRLs. WM9 cells were transfected with the pRK5 vector or pRK5 expression construct of Flag-tagged PRL-2 using LipofectAMINE. The cells were used at 48 h posttransfection for measuring the effects of pentamidine on the PTPase activities of intracellular Flag-PRL-2.

Animal Studies. Athymic nude mice 6 weeks of age (Taconic Farms Inc.) were inoculated (s.c.) in the flanks with WM9 cells (4×10^6 cells/site, two sites/mice) on day 0. Starting on day 2, the mice were subjected to no treatment (Control) or treatment with pentamidine (0.25 mg/mouse, every 2 days) injected i.m. at the hip area. The treatment dose (~6–10 mg/kg; mouse body weight, ~25–40 g during the study period) was chosen based on the therapeutic dose of the drug (2–4 mg/kg; Ref. 16) and the known toxicity of the drug at doses of >50 mg/kg (47). Tumor volume was calculated using the formula for a prolate spheroid $V = 4/3 \pi a^2 b$; Ref. 48) and depicted as mean \pm SE ($n = 8$). Mouse viability and body weights were recorded weekly. H&E-stained tissue sections of internal organs and tissues at tumor inoculation sites of the mice were prepared and subjected to microscopic evaluation.

Detection of Induced Cellular Protein Tyrosine Phosphorylation. WM9 cells were untreated or treated with various amounts of pentamidine for 5 min at 37°C. Cells were lysed in cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl,

0.2 mM Na_3VO_4 , 20 mM NaF, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, 20 $\mu\text{g/ml}$ of aprotinin, and 1 mM of sodium molybdic acid]. Cell lysates were separated in 10% SDS-PAGE gels, transferred to nitrocellulose membrane (Schleicher & Schuell), probed with specific antibodies, and detected using an enhanced chemiluminescence kit (ECL; Amersham).

RT-PCR Analysis of the Expression Levels of PRL Phosphatases. Expression of the transcripts of endogenous PRLs in PBMCs from two healthy volunteers and in cancer cells lines were detected by RT-PCR with specific primer pairs for individual PRLs, as listed below, or for GAPDH. RT-PCR products were separated in an agarose gel and visualized by ethidium bromide staining with their identities confirmed by restriction endonuclease mapping. The sequence of primer pairs are: huPRL-3/5, 5'-TAGGATCCCGGGAGGCGCCATGGCTCGGATGA-3'; huPRL-3/3, 5'-GAGTCCGACATAACGCAGCACCGGGTCTTGTG-3'; huPRL-2/5, 5'-TAGGATCCCCATAATGAACCGTCCAGCCCCCTGT-3'; huPRL-2/3, 5'-GAGTCGACCTGAACACAGCAATGCCCATTTG-3'; huPRL-1/5, 5'-TAGGATCCCCAACATGGCTCGAATGAACCGCCC-3'; and huPRL-1/3, 5'-GAGTCGACTTGAATGCAACAGTTGTTTCTATG-3'.

Results

Pentamidine Inhibits Selective Recombinant PTPases *in Vitro* with Its PTPase Specificity Different from SSG.

The activity of recombinant PTP1B in dephosphorylating a phosphotyrosine peptide *in vitro* was reduced in the presence of pentamidine in a concentration-dependent manner with nearly complete inhibition occurring at 1 $\mu\text{g/ml}$ of pentamidine (Fig. 1A). The inhibition of PTP1B by pentamidine *in vitro* was not abolished by a washing process (Fig. 1B), which was effective in reversing the inhibition of SHP-1 by suramin (27) and also detectable in PTPase assays using an alternative substrate (DiFMUP; Fig. 1C). In contrast, pentamidine showed little activity against recombinant SHP-1 and SHP-2 (Fig. 1, D and E). The activity of recombinant MKP1 was partially inhibited by pentamidine (Fig. 1F). Under comparable conditions, SSG inactivated SHP-1/SHP-2 at ~10 $\mu\text{g/ml}$ and inhibited PTP1B at a higher concentration (Fig. 1), which was consistent with our previous report (27).

These results demonstrate that pentamidine had inhibitory activity against selective PTPases *in vitro* with a specificity profile different from that of SSG.

Inhibition of Recombinant and Intracellular PRL Phosphatases by Pentamidine.

Given the potential pathogenic role of overexpression of PRLs in human malignancies (10, 11), these oncogenic phosphatases are highly attractive targets for developing inhibitors as novel anticancer therapeutics. Detection of an inhibitory activity of pentamidine against selective PTPases *in vitro* (Fig. 1) prompted us to investigate the effects of the drug on the PRL phosphatases.

The activities of recombinant PRL-1, PRL-2 and PRL-3 in dephosphorylating a phosphotyrosine peptide substrate *in vitro* were inhibited in the presence of pentamidine in a concentration-dependent manner with nearly complete inhibition of the phosphatases at 10 $\mu\text{g/ml}$ (Fig. 2A). Because the PRLs were affected similarly by pentamidine, PRL-3 was

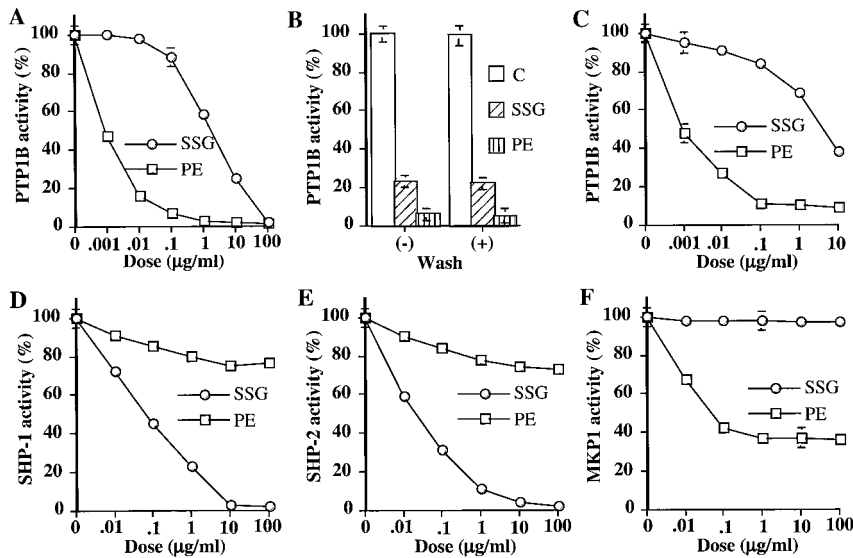
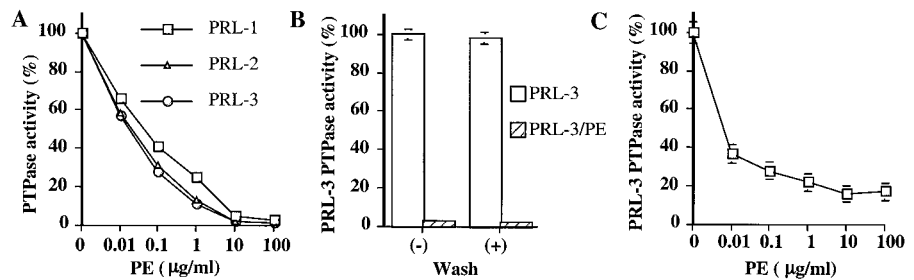


Fig. 1. Differential inhibitory activities of pentamidine (PE) against recombinant PTPases *in vitro*. Activities of GST fusion proteins of PTP1B (A), SHP-1 (D), SHP-2 (E), MKP1 (F) in dephosphorylating a phosphotyrosine peptide in the absence or presence of various amounts of PE or SSG were measured in *in vitro* PTPase assays. Relative activities of GST/PTP1B fusion protein untreated (control = C), preincubated with PE (1 µg/ml) or SSG (10 µg/ml) and then washed (+) or not washed (-) were determined using the peptide substrate (B). Activities of GST/PTP1B fusion protein in dephosphorylating DiFMUP in the absence or presence of PE or SSG were also determined by PTPase assays (C). Data represent mean ± SD values of triplicate samples.

Fig. 2. Pentamidine (PE) inhibits recombinant PRL phosphatases *in vitro*. A, relative activities of GST fusion proteins of PRLs in dephosphorylating a phosphotyrosine peptide substrate in the absence or presence of PE in *in vitro* PTPase assays. B, relative activities of GST/PRL-3 fusion protein preincubated with PE and then washed (+) or not washed (-) were determined using the peptide substrate. C, relative activities of GST-PRL-3 in dephosphorylating DiFMUP in the absence or presence of PE in PTPase assays. Data represent mean ± SD values of triplicate samples.



selected for further characterizing the inhibition induced by pentamidine. Recombinant PRL-3, preincubated with pentamidine and then subjected to a washing process, failed to dephosphorylate the peptide substrate (Fig. 2B), suggesting an irreversible action of the drug. The activity of recombinant PRL3 in dephosphorylating an alternative substrate (DiFMUP) was reduced in the presence of pentamidine (Fig. 2C). These results demonstrated that pentamidine had inhibitory activity against recombinant PRLs *in vitro*.

The effects of pentamidine against intracellular PRL phosphatases were assessed. To circumvent the difficulty of lacking mono-specific antibodies against individual PRLs, stable NIH3T3 transfectants of the control vector or expression constructs of PRLs tagged with the Flag epitope (38) were established. The transfectants were untreated or were treated with pentamidine for 5 min, washed to remove cell-free drug, and lysed for immunoprecipitation assays using an anti-Flag monoclonal antibody. A Flag-tagged protein of $M_r \sim 23,000$, as expected for Flag-PRL-1, was detected in the immunocomplexes from the Flag-PRL-1 transfectant (Fig. 3B, Lane 4) but not in those from vector control cells (Fig. 3B, Lane 1). The immunocomplexes from Flag-PRL-1 transfectant showed significant activities in dephosphorylating a synthetic phosphotyrosine peptide in PTPase assays (Fig. 3A, Lane 4), whereas those from vector control cells lacked such

an activity (Fig. 3A, Lane 1), demonstrating that the Flag-PRL-1 protein from the transfectant was an active PTPase. Interestingly, the immunocomplexes from pentamidine-treated Flag-PRL-1 transfectant failed to dephosphorylate the substrate (Fig. 3A, Lanes 5 and 6), although they contained Flag-PRL-1 protein at levels similar to those from the untreated cells (Fig. 3B, Lanes 5 and 6). Immunocomplexes from pentamidine-treated Flag-PRL-2 (Fig. 3C) or Flag-PRL-3 (Fig. 3E) also lacked PTPase activity in comparison with those of the untreated transfectants, despite approximately equal amounts of Flag-tagged PRLs in the samples (Fig. 3, D and F). These results demonstrated that pentamidine was an effective inhibitor against the PRLs ectopically expressed in the transfectants.

To assess the duration of pentamidine-induced inactivation of PRLs, the effects of pentamidine on one of the PRLs in the transfectants were further evaluated because the drug acted against the phosphatases in a similar manner (Figs. 2A and 3). The Flag-PRL-2 transfectant was treated with pentamidine (1 µg/ml) for 5 min, washed to remove cell-free drug, and then incubated for various times. Flag-PRL2, immunoprecipitated from transfectants that were incubated for 24 h posttreatment, showed relative PTPase activity of only 24% in comparison with that from the untreated cells (Fig. 4, A and C). PRL-2 from cells that were incubated for 48 or 72 h after

Fig. 3. Pentamidine (PE) inactivates intracellular PRLs ectopically expressed in NIH3T3 transfectants. **A**, PTPase activities of anti-Flag immunocomplexes from untreated (0) or PE-treated (5 min) NIH3T3 transfectants of the control vector (V) or Flag-PRL-1 expression construct in PTPase assays. **B**, relative amounts of Flag-PRL-1 in the immunocomplexes in (A) as detected by SDS-PAGE/Western blotting. **C**, PTPase activities of anti-Flag immunocomplexes from untreated or PE-treated NIH3T3 transfectants of Flag-PRL-2. **D**, relative amounts of Flag-PRL-2 in the immunocomplexes as determined by SDS-PAGE/Western blotting. **E**, PTPase activities of anti-Flag immunocomplexes from untreated or PE-treated NIH3T3 transfectants of Flag-PRL-3. **F**, relative amounts of Flag-PRL-3 in the immunocomplexes as determined by SDS-PAGE/Western blotting. Data of the phosphatase assays represent mean \pm SD values of triplicate samples.

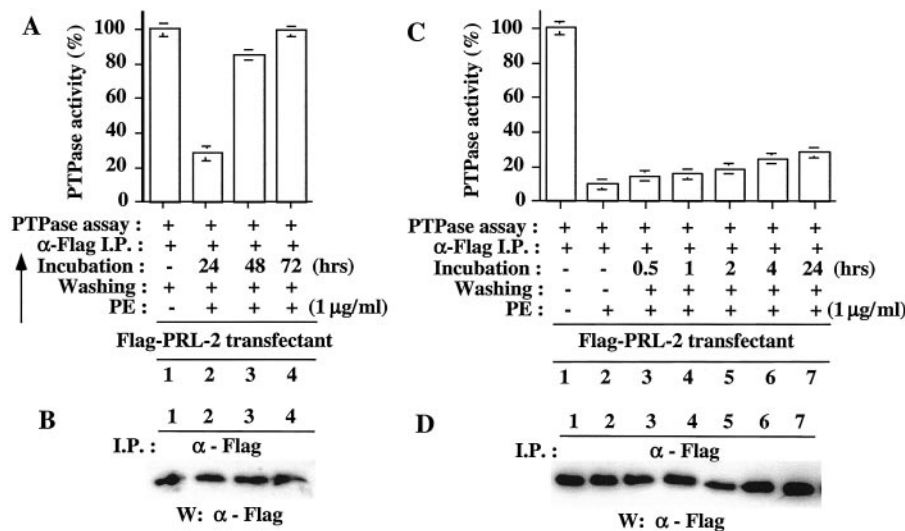
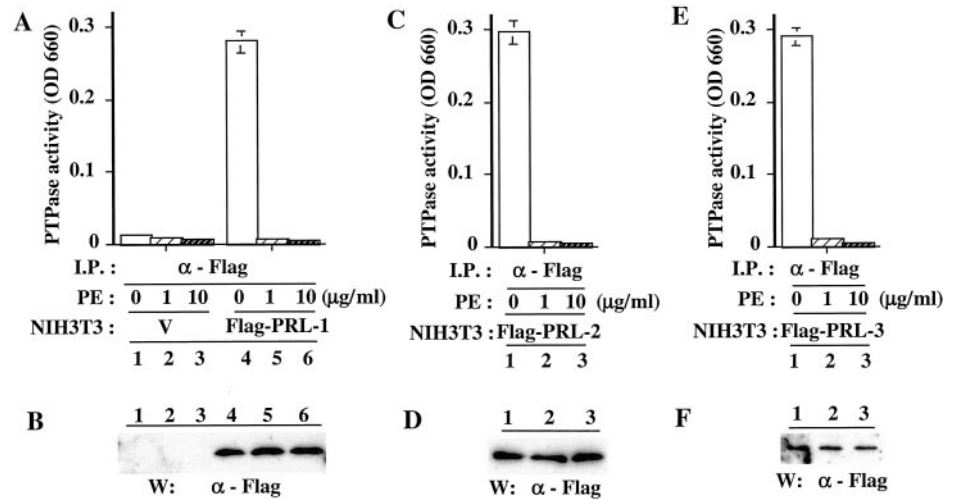


Fig. 4. Duration of pentamidine (PE)-induced inactivation of intracellular PRL-2 in NIH3T3 transfectants. **A** and **C**, relative PTPase activity of anti-Flag immunocomplexes from NIH3T3 transfectant of Flag-PRL-2 untreated or treated with PE for 5 min, washed to remove cell-free drug and then incubated for various times before termination by lysing the cells. Data represent mean \pm SD values of triplicate samples. **B** and **D**, relative amounts of Flag-PRL-2 in the immunocomplexes as determined by SDS-PAGE/Western blotting.

pentamidine treatment showed relative PTPase activities of 86 or 98%, respectively (Fig. 4A). The amounts of PRL-2 protein in the immunocomplexes from the treated or untreated cells were comparable (Fig. 4, B and D). Thus, brief pentamidine treatment had an inhibitory effect on the ectopically expressed PRL-2 that required more than 48 h for its complete removal.

Pentamidine Inhibits the *in Vitro* Growth of Human Cancer Cell Lines Expressing Endogenous PRLs. The inhibitory activity of pentamidine against PRLs suggested the potential of the drug against cancer cells expressing these oncogenic phosphatases. We, therefore, determined the effects of the drug on the *in vitro* growth of human cancer cell lines and assessed the expression of PRLs in these cells. The cell lines were derived from different human malignancies, including melanoma (WM9), prostate carcinoma (DU145 and C4-2), ovarian carcinoma (Hey), colon carcinoma (WM480), and lung carcinoma (A549).

The growth of all six of the cell lines in culture was inhibited by pentamidine in a concentration-dependent manner with complete growth inhibition of the cell lines occurring at 10 μ g/ml (Fig. 5), as confirmed by the absence of viable cells under microscopic examination (data not shown). The drug also showed significant growth-inhibitory effects at lower concentrations (0.3–5 μ g/ml) that were similar to its therapeutic dosage (2–4 mg/kg body weight; Ref. 16). Among the cell lines, A549 cells were most sensitive to the drug with 45 and 94% growth inhibition achieved at 0.3 μ g/ml and 2.5 μ g/ml, respectively (Fig. 5C). Although the most resistant cell line SW480 was barely affected by the drug at 0.3 μ g/ml, the growth of the cell line was significantly inhibited (74%) by pentamidine at 2.5 μ g/ml (Fig. 5E). The other cell lines showed pentamidine sensitivities falling between those of A549 and SW480 (Fig. 5). RT-PCR analysis revealed the presence of the transcripts of the PRLs in the cell lines, with

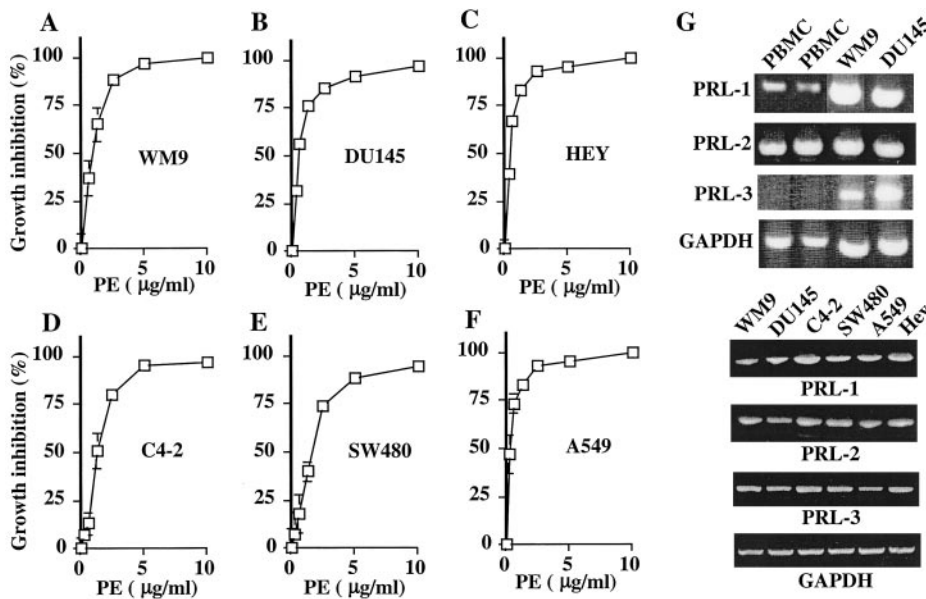


Fig. 5. Pentamidine (PE) inhibits *in vitro* growth of human cancer cell lines that express endogenous PRLs. A-F, growth of cell lines of different human malignancies cultured in the absence or presence of various amounts of PE for 6 days was determined by MTT assays. Data represent mean \pm SD values of triplicate samples. G, expression of transcripts of PRLs in the cell lines and in PBMCs from two healthy volunteers as determined by RT-PCR.

PRL-1 and PRL-3 expression at levels higher than those in the PBMCs of two healthy volunteers (Fig. 5G).

These results demonstrated an *in vitro* growth-inhibitory activity of pentamidine against different human cancer cell lines that expressed the endogenous PRL phosphatases.

Pentamidine Inhibits the Growth of WM9 Human Melanoma Tumors in Nude Mice at a Tolerable Dose. To further assess the anticancer potential of pentamidine, the effects of the drug on the growth of WM9 tumors in nude mice were evaluated.

WM9 cells inoculated in nude mice formed aggressively growing tumors (Fig. 6A), consistent with a previous report (49). The growth of WM9 tumors was markedly inhibited by pentamidine treatment (250 μ g/mouse, every 2 days; Fig. 6A). During the 16-week study period, the tumors in pentamidine-treated mice stayed at sizes similar to those at the treatment initiation point, whereas the tumors in the control mice grew so rapidly that humane sacrifice of the animals was required at the 4th week (Fig. 6A). This pentamidine treatment caused no obvious abnormalities in the mice, which all survived and showed steady body weight gains during the study period (data not shown). The histology of internal organs (heart, kidney, liver, lung, and spleen) of the pentamidine-treated mice at the end of the study period was unremarkable (data not shown). Evaluation of the tumors in these mice revealed significant necrosis that accounted for more than 50% of the tumor mass (Fig. 6, B and C), which was absent in tumors from untreated control mice (Fig. 6B).

Thus, pentamidine at a tolerable dose effectively inhibited the growth of WM9 tumors in nude mice and induced extensive tumor cell necrosis.

Pentamidine Inactivates PRL-2 and Augments Cellular Protein Tyrosine Phosphorylation in WM9 Cells. In light of the growth-inhibitory activity of pentamidine against WM9 tumors in nude mice, we further investigated whether pentamidine functioned as an inhibitor of PRLs in WM9 cells.

Because the three PRLs were similarly inhibited by the drug in NIH3T3 cells, the effects of pentamidine of one of the PRLs in the cancer cells were evaluated.

WM9 cells were transiently transfected with an expression construct of Flag-PRL-2 or the control vector. The transfectants were untreated or treated with pentamidine for 5 min, washed, and lysed for immunoprecipitation assays using an anti-Flag antibody. A Flag-tagged protein of $M_r \sim 23,000$, as expected for Flag-PRL-2, was detected in the immunocomplexes from Flag-PRL-2 transfectant but not in those from the vector control cells (Fig. 7A). The immunocomplexes from the untreated Flag-PRL-2 transfectant showed significant activity in dephosphorylating the phosphotyrosine peptide substrate whereas the ones from pentamidine-treated Flag-PRL-2 transfectant had activity similar to those of the vector control cells (Fig. 7B), demonstrating inactivation of the phosphatase in pentamidine-treated WM9 cells. In contrast, SHP-2 immunoprecipitated from untreated or pentamidine treatment of WM9 cells had comparable PTPase activities (Fig. 7C and D), indicating that this phosphatase in WM9 cells was insensitive to inhibition by pentamidine as recombinant SHP-2 was *in vitro* (Fig. 1). Treatment of WM9 cells with pentamidine for 5 min resulted in increased tyrosine phosphorylation in several cellular proteins yet to be identified (Fig. 7E), consistent with the inhibition of PTPases in the cancer cells by the drug.

These results demonstrated that pentamidine functioned as an inhibitor that selectively inhibited ectopically expressed intracellular PRL-2 but not the endogenous SHP-2 and induced cellular protein tyrosine phosphorylation in WM9 melanoma cells.

Discussion

Pentamidine and SSG have common features in their action against leishmaniasis. Our recent finding that SSG might

Fig. 6. Pentamidine (PE) inhibits the growth of WM9 human melanoma tumors in nude mice coincident with tumor cell necrosis. **A**, tumor volumes in nude mice inoculated with WM9 cells (s.c.) at the flanks and subjected to no treatment (*Control*) or treatment with PE (0.25 mg/mouse, i.m., every 2 days at the hip area) were measured on the dates as indicated. Data represent mean \pm SE ($n = 8$). **B**, representative views ($\times 4$) of H&E-stained sections of WM9 tumors in nude mice without treatment at the 4th week after inoculation (*left*) and WM9 tumors in nude mice treated with PE for 16 weeks (*right*). **C**, a higher power view ($\times 40$) of the tumor at the right side of **B**.

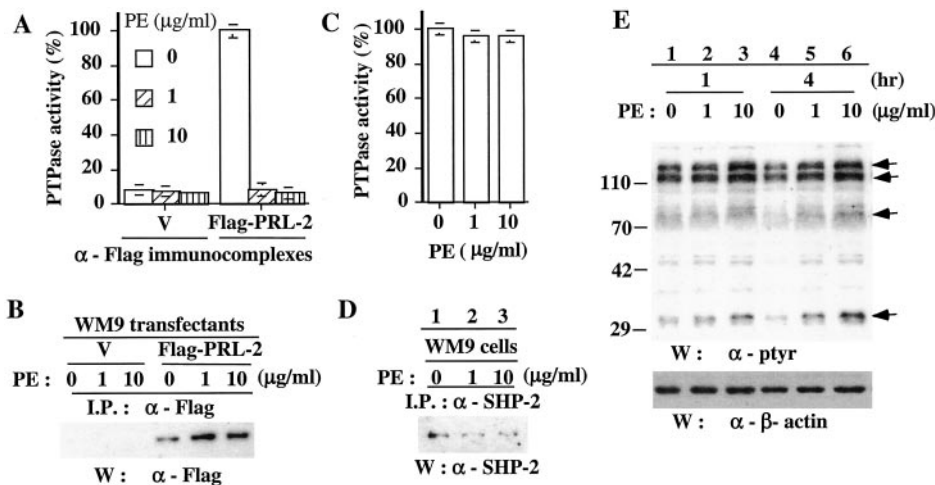
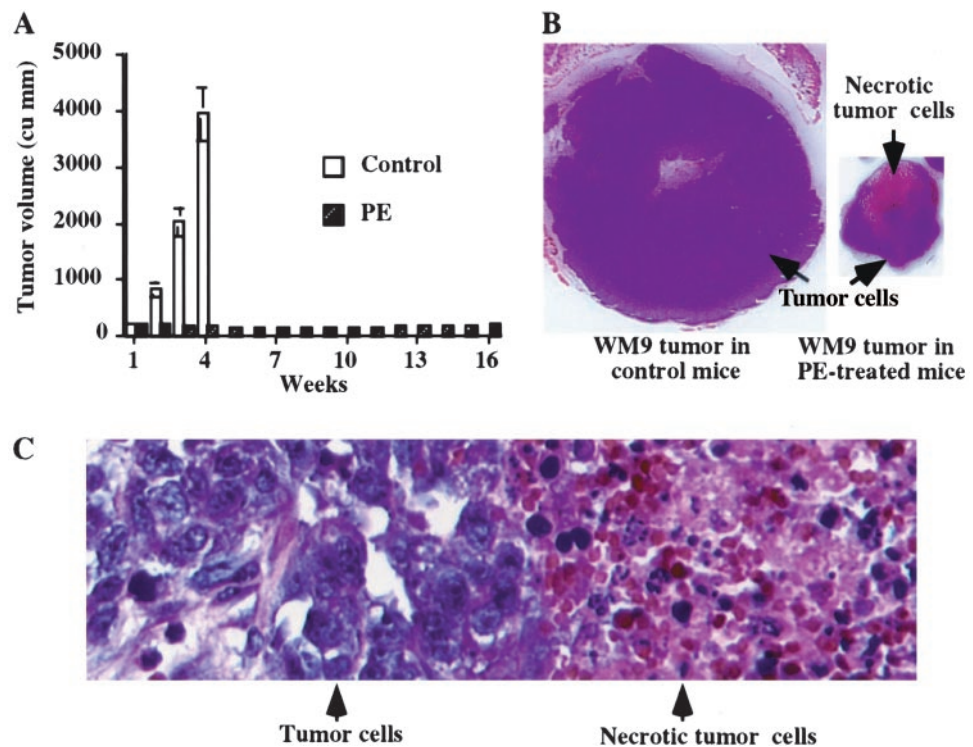


Fig. 7. Pentamidine (PE) inhibits intracellular PRL-2 ectopically expressed in WM9 cells and induces cellular protein tyrosine phosphorylation. **A**, relative PTPase activities of anti-Flag immunocomplexes from WM9 cells transfected with the control vector (V) or Flag-PRL-2 expression construct and then treated with different amounts of PE for 5 min. Data represent mean \pm SD values of triplicate samples. **B**, relative amounts of Flag-PRL-2 in the immunocomplexes as determined by SDS-PAGE/Western blotting. **C**, relative PTPase activities of anti-SHP-2 immunocomplexes from WM9 cells treated with different amounts of PE for 5 min. Data represent mean \pm SD values of triplicate samples. **D**, relative amounts of SHP-2 in the immunocomplexes as determined by SDS-PAGE/Western blotting. **E**, total cell lysates of WM9 cells treated with different amounts of PE were analyzed by SDS-PAGE/Western with antibodies as indicated. *On the left*, the positions of protein size markers, M_r in thousands. *Arrows*, several phosphotyrosine cellular proteins affected by PE.

function via inhibiting negative regulatory SHP-1 PTPase to augment signaling and leishmanicidal activity of host cytokines suggests the possibility that pentamidine may function in a similar manner as a PTPase inhibitor.

Indeed, pentamidine was an effective inhibitor of PTP1B. Pentamidine at 1 μ g/ml resulted in nearly complete inhibition of recombinant PTP1B in dephosphorylating a peptide sub-

strate (Fig. 1A) or a fluorescent substrate (Fig. 1C). This pentamidine concentration is likely achievable *in vivo* based on its tissue disposition (1.6–34 μ g/g) in rats at 24 h after pentamidine injection (4 mg/kg; Ref.50) and its tissue concentrations in patients treated with the drug (51). It suggests that inhibition of PTP1B might occur *in vivo* during pentamidine therapy. Because PTP1B dephosphorylates and inac-

tivates Jak kinases (35), which mediate signaling of cytokines with leishmanicidal activity, its inhibition by pentamidine might result in augmentation of cytokine signaling and anti-leishmania effects. Interestingly, pentamidine showed only minor effects on PTPase activity of SHP-1 (Fig. 1D), which was inhibited by SSG under comparable conditions (Fig. 1D). These results together suggest that pentamidine and SSG might target different negative regulatory PTPases to mediate their anti-leishmania action, and probably other pathogens, through augmenting cytokine signaling.

Significantly, we demonstrate herein for the first time that pentamidine is also a potent inhibitor of the oncogenic PRL phosphatases. The drug at 1–10 $\mu\text{g/ml}$ effectively inhibited recombinant PRLs *in vitro* (Fig. 2). Moreover, intracellular PRLs from NIH3T3 transfectants pulse treated with pentamidine (1 or 10 $\mu\text{g/ml}$) were inactivated (Fig. 3) and required more than 48 h for their full recovery (Fig. 4). The drug also inactivated PRL-2 ectopically expressed in WM9 melanoma cells (Fig. 7B), demonstrating its effectiveness against the phosphatase in human cancer cells. The inhibitory activity of the drug was restricted to a subset of PTPases in cancer cells because pentamidine failed to inactivate the endogenous SHP-2 PTPase in WM9 cells (Fig. 7D). The fact that recombinant SHP-2 was also insensitive to the drug *in vitro* (Fig. 1E) suggests a correlation of *in vitro* and *in vivo* sensitivities of PTPases to the drug and supports the notion that the *in vitro* sensitive PTP1B (Fig. 1A) might also be a target of pentamidine *in vivo*.

One of the most interesting findings of our studies is the demonstration that pentamidine at tolerable doses had marked growth-inhibitory activity against human cancer cell lines *in vitro* and in a xenograft mouse model. Pentamidine, at ~6–10 mg/kg, induced complete growth inhibition of WM9 melanoma tumors in nude mice during the 16-week study period (Fig. 6A). This is striking in comparison with the aggressive growth of WM9 tumors in the untreated mice that resulted in the termination of the animals 4 weeks after tumor inoculation (Fig. 6A). The dosage used in this study is similar to the therapeutic dose of the drug (2–4 mg/kg; Ref. 16) and did not result in obvious toxicity in the animals. Given that pentamidine inhibited the growth of cell lines of several other human malignancies in culture (Fig. 5), the drug might have activity against different types of cancers. Additional studies to assess the efficacy of pentamidine on more established tumors and tumors of different tissue types will provide additional insights into the anticancer potential of the drug.

The anticancer activity of pentamidine is likely mediated via inactivation of cancer cell-expressed PTPases, in particular the oncogenic PRLs, and resulted in preferential killing of the malignant cells. Like the transfected PRLs in NIH3T3 fibroblasts (Fig. 3), PRL-2 ectopically expressed in WM9 melanoma cells (Fig. 7) were inactivated by pentamidine at 1 $\mu\text{g/ml}$, a dosage within its tissue levels (1.6–34 $\mu\text{g/g}$) in rats at 24 h after pentamidine injection (4 mg/kg; Ref. 50). Thus inactivation of PRLs might occur in WM9 tumor cells in the pentamidine-treated mice. In light of the detected expression of endogenous PRLs in WM9 cells (and the other cancer cell lines; Fig. 5) and the known oncogenic activity of the phosphatases (5, 12), PRLs might be among the key targets of the drug in mediating its anticancer activity. Thus, human ma-

lignancies overexpressing the PRL phosphatases might be sensitive to pentamidine therapy, and PRLs may serve as markers for identification of pentamidine-sensitive tumors. In this regard, the duration of pentamidine-induced inactivation of PRL-2 could be important because it provides a basis for rational design of PRL-targeted pentamidine therapy in cancer treatment. Although PTP1B is also a potential pentamidine-target PTPase and might mediate the drug's anti-leishmania effects, its significance in cancer cell growth inhibition by pentamidine is less clear given the negative role of the PTPase in signaling. Additional studies to evaluate pentamidine activity against PRL-negative cancer cells will help to define the putative role of PRLs in mediating the antitumor effects of the drug.

Our finding that pentamidine is an inhibitor of selective PTPases is also significant in developing novel PTPase-targeted therapeutics. Because pentamidine is a chemically defined compound with a number of derivatives already reported (52, 53), screening such derivatives might lead to mono-specific inhibitors against individual PRLs or other PTPases. It also allows structural analysis of target PTPases in complex with pentamidine, which could provide a basis for rational design of the next generations of more specific inhibitors against these oncogenic phosphatases. Furthermore, the effectiveness of pentamidine in inactivating PRLs in cancer cells suggests its potential value as an experimental tool in elucidating the physiological function and oncogenic mechanism of PRLs.

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