

Hepoxilin Analogs, PBT-3 and PBT-4, Cause Apoptosis of Gleevec-resistant K562 Cells *In Vitro*

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Abstract. *The use of Gleevec® in the treatment of leukemia has been widely accepted, although resistance to Gleevec is commonly observed. Gleevec represents a new direction in the development of target-focused chemotherapeutic agents in cancer. Gleevec inhibits the tyrosine kinase activity of Bcr-Abl, which is responsible for leukemic cell survival. We have previously shown that PBT-3 (racemic anti-10(R/S)-hydroxy-11,12-cyclopropyl-eicosa-5Z, 8Z,14Z-trienoic acid methyl ester) and PBT-4 (racemic syn-10(R/S)-hydroxy-11,12-cyclopropyl-eicosa-5Z 8Z,14Z-trienoic acid methyl ester), stable analogs of the hepoxilins, caused apoptosis of the human leukemic K562 cell line in vitro and in vivo. We also showed that PBTs inhibited the growth of tumours derived from the inoculation of immunodeficient mice with K562 cells and that the effect of PBTs was synergistic with that of Gleevec. We now show that the effect of PBT-3 and of PBT-4 is independent of that of Gleevec, demonstrating that Gleevec-resistant K562 cells retain their responsiveness to PBT treatment, resulting in apoptosis. These findings provide important information suggesting that the two compounds, PBT and Gleevec, can be used together in the treatment of leukemia. The PBTs may provide a new platform for the development of apoptotic drugs in cancer.*

Leukemia is a heterogeneous disease characterized by malignant proliferation of cells of the hematopoietic system. Leukemic cells contain a Bcr-Abl chimeric fusion protein, the result of a reciprocal translocation between chromosome 9 and chromosome 22 referred to as the Philadelphia (Ph) chromosome. Bcr-Abl expression is considered a high-risk

factor due to the poor treatment outcome (1, 2). Bcr-Abl has increased levels of tyrosine kinase (TK) activity and plays a role for increased leukemogenesis. The effective use of K562 cells as an *in vitro* model for leukemia studies is due to its expression of Bcr-Abl protein (3). K562 cells were derived from a Chronic myelogenous leukemia patient in blast crisis. Furthermore, K562 cells are resistant to apoptosis induced by chemotherapeutic agents. The anti-apoptotic effects of Bcr-Abl can be overcome by inhibiting its expression (4, 5) and/or its tyrosine kinase activity (6, 7). Gleevec has been hailed as a novel targeted drug for the treatment of leukemia (8). It targets tyrosine kinase (TK), an enzyme in leukemic cells that is responsible for the resistance of these cells to apoptosis (9). Apoptosis induction by Gleevec was associated with release of cytochrome c from the mitochondria and activation of downstream caspase (10) indicating that it can reverse the inhibitory effect of Bcr-Abl on the mitochondria.

Hepoxilins (*syn*- and *anti*-8-S/R-hydroxy-11,12-epoxy-5Z,9E,14Z-trienoic acid and *syn*- and *anti*-10-S/R-11,12-epoxy-5Z,8Z,14Z-trienoic acid) are small molecule oxygenated lipids derived from arachidonic acid (11). We have shown that PBTs, stable analogs of the hepoxilins (12), inhibit K562 cell proliferation and induce apoptosis *in vitro* and *in vivo* (13, 14). Now we report on a study comparing the effects of PBT-3 (racemic *anti*-10(R/S)-hydroxy-11,12-cyclopropyl-eicosa-5Z, 8Z,14Z-trienoic acid methyl ester) and PBT-4 (racemic *syn*-10(R/S)-hydroxy-11,12-cyclopropyl-eicosa-5Z 8Z,14Z-trienoic acid methyl ester) with those of Gleevec in parental K562 cells and in Gleevec-resistant K562 cells to determine whether the PBTs inhibit cell growth and promote cytochrome c release independent of the mechanism operated by Gleevec.

Materials and Methods

RPMI-1640, fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), phosphate-buffered saline (PBS), trypan blue and trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA) were purchased from Wisent Inc. (St. Bruno, Quebec, Canada). [Methyl-³H]-thymidine (25 Ci/mmol), was purchased from Amersham Life Science (Baie d'Urfe, Quebec, Canada), Dimethylsulfoxide (DMSO)

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was purchased from Caledon (Georgetown, Ontario, Canada). The heptoxilin analogs, PBT-3 and PBT-4, were prepared in our laboratory by total chemical synthesis as previously described (12). Gleevec was from Novartis Pharma was obtained in pill form from our Hospital's pharmacy. Cell proliferation reagent WST-1 was purchased from Roche Applied Science (Laval, Quebec, Canada). All other chemicals and reagents were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

Cell culture. Human leukemia K562 cells, obtained from the Hematology Department of Hospital for Sick Children, were maintained as suspension cultures in RPMI-1640 medium containing 100 U/ml penicillin G, 100 µg/ml streptomycin, 10% (v/v) FBS in a humidified atmosphere of 5% CO₂ at 37°C. To avoid cell overgrowth in the experiments that measured the effect of different times of PBT exposure, cells were collected, counted and resuspended in fresh medium at lower cell density, and the cell numbers were normalized accordingly.

Cell viability assay. Cells were plated at a density of 5x10⁴ cells/ml in RPMI 1640 with or without Gleevec or PBT. The ability of the cells to exclude Trypan Blue dye was used to assess cell viability.

Generation of Gleevec-resistant cell lines. K562 cell lines maintained in liquid culture were initially exposed to 0.1 µM Gleevec and were gradually exposed to increasing concentrations of Gleevec at the rate of 0.1 µM increment every 10 days of culture. After approximately 2 months, sublines of cells growing in 1 µM Gleevec were maintained continuously in culture at this dose of the inhibitor. Parental K562 cells were maintained in parallel cultures without Gleevec to be used as controls. Studies showed the modified cells to be resistant to the effects of Gleevec in terms of [Methyl-³H]-thymidine incorporation, while the parental K-562 cells behaved in a normal way.

Thymidine incorporation. Tumor cell growth and inhibition assays. Incorporation of [Methyl-³H]-thymidine (50,000 dpm/tube) into cell DNA was measured as described elsewhere [2]. K562 cells, starved overnight (0.5% FBS), were treated with various doses (0-30 µM) of PBT-4 and Gleevec in 1% FBS serum. DMSO was used as vehicle. At 6 h post-treatment incubation at 37°C, cells were fixed and denatured, then harvested and passed through Whatman GF/C glass filter. Filters were washed and counted for radioactivity in a Beckman LS 3800 scintillation counter.

Cell proliferation assay. Cell proliferation assay was performed using WST-1 tetrazolium salt, which allows measurement of numbers of viable cells. Overnight starved cells (0.5% FBS), between 5x10³ and 5x10⁴, were washed twice in RPMI-1640 and plated in triplicate into microtiter-plate wells in 100 µl RPMI-1640 plus various doses of Gleevec and PBT-4 (0-55 µM). Control wells using the same concentrations of Gleevec or PBT-4 but without cells were set up in parallel. WST-1 (10 µl) was added to each well and the plates were incubated for 2 h at 37°C. The plates were read in a microplate autoreader (VERSAmix microplate reader; Sunnyvale, California, USA) at 450 nm wavelength, background absorbance was at 600 nm. Results are expressed as the mean optical density of the 3-well set for each Gleevec or PBT-4 dose. All experiments were repeated 3 times.

Western blotting of cytochrome c. Serum-starved (0.5% FBS) Gleevec-resistant K562 cells were treated with Gleevec with or without 2.8 µM PBT-3 for 24 h. Treatment was terminated by washing cells with ice-cold PBS buffer. Cell lysates were prepared in buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton[®] X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 µM leupeptin on ice for 60 min. The lysates were clarified by centrifugation at 15,000xg for 15 min at 4°C. Lysates were subjected to protein assay and kept at -80°C. Protein (250 µg) was immunoprecipitated with anticcytochrome c antibody coupled to protein A-agarose beads. After washing of the immunocomplexes with lysis buffer, SDS-PAGE sample loading buffer was added and the mixture was boiled for 5 min. After centrifugation, the supernatant was loaded onto 10-12% SDS-PAGE gel and transferred to a Trans-Blot Nitrocellulose membrane (Bio-Rad Laboratories Canada Ltd., Life Sciences Group, Mississauga, Ontario, Canada). Protein bands on the nitrocellulose membranes were checked visually with Ponceau S-staining to assure equivalent protein loading/transfer comparing different samples. Membranes were blocked with nonfat dry milk (5%, w/v) in PBS containing 0.5% (v/v) Tween-20 for one hour at room temperature and then incubated with 1:1000 dilution of anticcytochrome c antibody overnight at 4°C; secondary antibody of horseradish peroxidase anti-rabbit or anti-mouse antibody was used at 1:2000 dilution. Bound antibodies were detected using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences Inc., Baie d'Urfe, Quebec, Canada) and the membranes were exposed to Hyperfilm for ECL. Tubulin antibody was used to ensure equivalent sample loading.

Statistical analysis. The observed differences in thymidine incorporation and cell proliferation assay were analyzed using an unpaired double-factor analysis of variance test with StatView (BrainPower Inc., Calabasas, California, USA).

Results

Effect of PBT-4 on [Methyl-³H]-thymidine incorporation in the leukemia cell lines, parental K562 and Gleevec-resistant K562 cells. Figure 1 shows the dose-dependence of both cell lines when treated with PBT-4 or Gleevec. Dimethylsulfoxide was used as a vehicle control. As the dose of PBT-4 increased, incorporation of [Methyl-³H]-thymidine into the cells decreased. Increasing the dose of PBT-4 from 1 µM to 2.8 µM produced the greatest difference in thymidine incorporation on both the K562 and the Gleevec-resistant K562 (Gleevec-Re-K562) cells [IC₅₀=2.8 µM]; at a dose of 7 µM of PBT-4, thymidine incorporation was reduced to 90% of control, and further increasing the PBT-4 dose to 14 µM had no more effect on either cell type. Increasing doses of Gleevec of 10 to 20 µM caused a reduction in thymidine incorporation from 40% to 80% when parental K562 cells were used [IC₅₀=12 µM]. In contrast, Gleevec has minimal effect in Gleevec-Re-K562 cells, *i.e.* at a dose of Gleevec from 5 µM to 20 µM, thymidine incorporation decreased only 10%

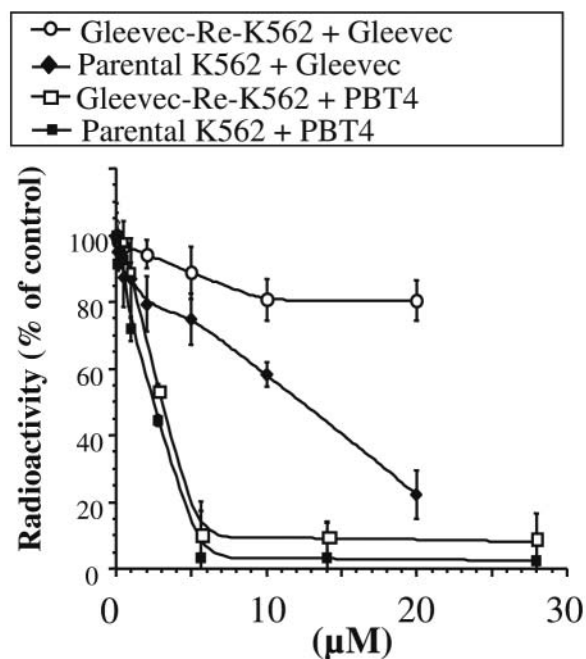


Figure 1. Dose-dependence of PBT-4 and Gleevec on [Methyl-³H]-thymidine incorporation in parental K562 cells and Gleevec-resistant-K562 cells (Gleevec-Re-K562 cells). Values are expressed as a mean of 5 separate cultures carried in duplicates.

compared with the DMSO control. These data indicated strong inhibition by PBT-4 in both parental K562 and Gleevec-Re-K562 cell lines, while Gleevec inhibited thymidine incorporation only in the parental K562 but not in Gleevec-Re-K562 cell lines.

Inhibitory effects of PBT-4 and Gleevec on parental K562 and Gleevec-Re-K562 cell proliferation. Figure 2 shows the dose-dependence of PBT-4 in both cell types indicating that the concentration of PBT-4 needed for a 50% reduction in the number of viable cells after exposure to the WST was 7 µM; at a dose range of 7 µM to 28 µM, the rate of proliferation was only reduced by an additional 10%. In contrast to PBT-4, Gleevec induced significant differences in growth inhibition between K562 and Gleevec-Re-K562 cells. Gleevec strongly inhibited cell growth in parental K562 cells even at 1 µM, while at 10 µM of Gleevec, reduction amounted to little more than 50%. Between 20 µM to 40 µM of Gleevec, parental K562 cell growth was inhibited by 60% to 90%. In contrast, Gleevec-Re-K562 cells showed minimal response to doses of Gleevec from 1 µM to 20 µM. These data support the data in Figure 1 showing the response of both cell lines (parental and Gleevec-Re-K562) to PBT-4 while Gleevec affects only parental K562 cells.

Western blot – cytochrome c analysis in Gleevec-resistant K562 cells. In order to investigate whether cytochrome c release is intact in the Gleevec-Re-K562 cells and to confirm that Gleevec is ineffective in the Gleevec-Re cells, we carried out a study in which these cells were incubated in the presence of Gleevec (control), PBT-3, and a combination of both drugs, and measured cytochrome c release from the mitochondria and its appearance in the cytosol. Figure 3 shows the results. Gleevec was mostly ineffective in releasing cytochrome c from the mitochondria (pellet), while PBT-3 was greatly effective. The combination of both drugs is really a representation of the effect of PBT-3. These results confirm the apoptotic effect of PBT in Gleevec-resistant K562 cells and demonstrate that: i) the cytochrome c release mechanism is still intact in these cells; and ii) that PBT induces apoptosis through the activation of the release of cytochrome c as previously shown in parental K562 cells (13).

Discussion

Recent reports have documented several promising strategies that target either the mRNA or protein encoded by the Bcr-Abl fusion gene, which is pathogenetically responsible for the malignant phenotype of CML and Bcr-Abl-positive adult acute lymphoblastic leukemia (ALL). Specific inhibitors of Bcr-Abl TK have undergone more comprehensive *in vitro* and *in vivo* evaluation. Among these, Gleevec has already been shown not only to induce apoptosis of Bcr-Abl-positive leukemic cells but also to display promising clinical activity against CML and adult ALL (3). There are several molecular mechanisms that have been implicated as being involved in Bcr-Abl-mediated resistance to the intrinsic or mitochondria pathway to apoptosis. Our finding demonstrates that PBT-4 strongly inhibits cell proliferation in Gleevec-Re-K562 cells, while Gleevec action is reduced in these cells. The mechanism of inhibition of cell growth and induction of apoptosis in parental K562 cells by PBT-3 was demonstrated in our previous study, *i.e.* PBT caused procaspase-3 cleavage and enhanced cytochrome c release from the mitochondria. Herein we show that PBT-3 retains its ability to enhance the release of cytochrome c from the mitochondria in the Gleevec-resistant cell line while response to Gleevec is suppressed, supporting our finding that PBTs retain their apoptotic action in the Gleevec-resistant cell line.

Resistance to chemotherapy is the major obstacle in the treatment of cancer. For the majority of anti-cancer drugs, apoptosis appears to be initiated by the intrinsic mitochondrial, or cytochrome c/caspase-3 pathway. Alternatively, this pathway could play a role in drug

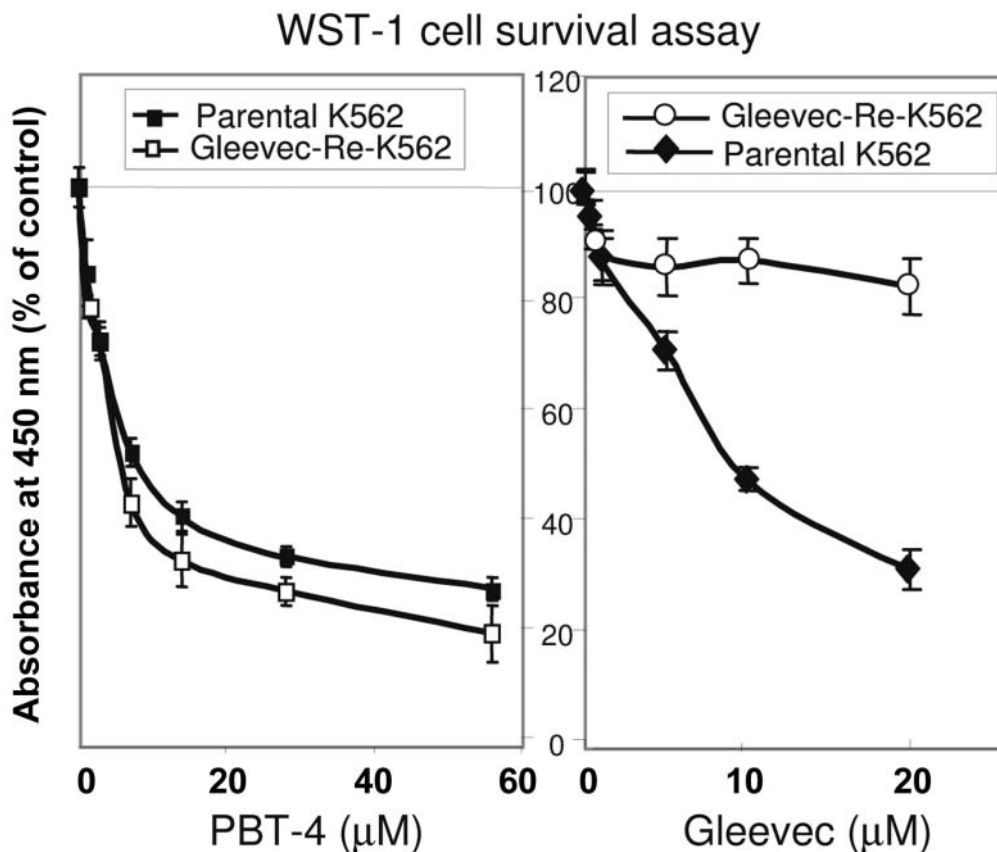


Figure 2. Cell viability of parental K562 cells and Gleevec-resistant K562 cells, i.e. Gleevec-Re-K562, as assessed in the WST-1 assay. Results are expressed as the mean OD 450 nm of triplicate cultures; this is directly proportional to the number of viable cells.

resistance (15). Gleevec has provided a novel drug for leukemia treatment. This molecule is in fact operationally specific for neoplasias caused by the oncogenic tyrosine kinase Bcr/Abl, and holds promise for minimal toxicity to normal cells when compared to available cytotoxic drugs (16). But there is still the question of acquired resistance to Gleevec. With PBT-4 or PBT-3 no drug resistance was found. With doses of PBT-4 over 7 μM (Figure 1), most of the cells undergo apoptosis.

Because of Gleevec resistance, biologically active cytotoxic molecules have been considered as potential new therapies in the destruction of drug-resistant tumor cells. Some members, TNFα, CD/95FasL have already been characterized (17). The PBTs, analogs of the natural mediators hepxilins, possess diverse functions including apoptosis of neoplastic cells, and anti-inflammatory and anti-thrombotic activities (18-20). The PBTs are active *in vivo* in mouse models of leukemia (K562 induced tumours) and function on their own and in synergy with Gleevec in controlling tumour growth *in vivo* (14). Hence, these compounds have features that may be ideal for development as novel therapeutics in cancer.

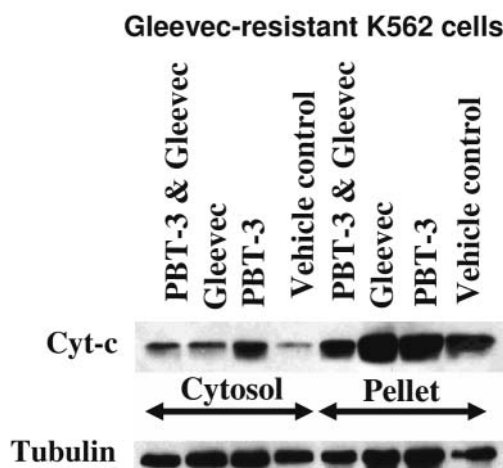


Figure 3. Western blot of Cytochrome c present in the mitochondrial (pellet) and cytosol fractions of Gleevec-Re-K562 cells treated with PBT-3 (28 μM), Gleevec (1 μM) or both to show that the cells still retain movement of Cytochrome c ONLY after treatment with PBT-3 but not Gleevec. Note presence of Cytochrome c in the mitochondria in control conditions (vehicle treated), and refractory release by Gleevec. However, a large release into the cytosol is observed with PBT-3. Tubulin detection serves as sample loading control.

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