

The Hepoxilin Analog, PBT-3, Inhibits Growth of K-562 CML Solid Tumours *In Vivo* in Nude Mice

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Abstract. PBT-3 is one of a family of stable chemical analogs of the hepoxilins, products derived from arachidonic acid. We previously showed that PBT-3 caused apoptosis in the chronic myelogenous leukemia (CML) cell line K-562 *in vitro* (*Anti-cancer Res* 23: 3617-3622, 2003). It was as effective as Gleevec, a novel agent that blocks tyrosine kinase activity during treatment of CML. We describe, herein, the growth inhibiting effects of PBT-3 in nude mice into which K-562 cells were transplanted subcutaneously. Groups of mice were treated with vehicle as control, or PBT-3, or Gleevec. PBT-3 was effective during the 8-day treatment protocol in inhibiting the growth of the tumours *in vivo* as was Gleevec. Analysis of the tumours demonstrated the presence of apoptosis (DNA laddering and TUNEL assay) in both the PBT-3- and Gleevec-treated groups. These results demonstrate that PBT-3 is effective *in vivo* in controlling tumour growth and provides a novel platform for the therapeutic control of cancer.

The hepoxilins are derived from arachidonic acid through the action of 12(S)-lipoxygenase (1-3). Four compounds are formed enzymatically, two epimers of hepoxilin A3, and two epimers of hepoxilin B3. Hepoxilins of the A3 series potently release intracellular calcium from human neutrophils (4), while those of the B3 series are mostly inactive. Despite this, all four hepoxilins are capable of inhibiting the release of intracellular calcium evoked by several unrelated inflammatory mediators including, fMLP, leukotriene B4 and PAF (5). As the native hepoxilins are unstable due to low pH and to metabolism by epoxide hydrolase (6-8) and glutathione S-transferase (9, 10), the compounds were not suitable for investigations *in vivo*.

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Hence, we designed compounds (PBTs) to mimic the effects of the native hepoxilins but to have stability for *in vivo* use (11). Indeed we have shown that the PBTs are effective *in vivo* in preventing the onset of lung fibrosis induced by bleomycin, a chemotherapeutic agent (12). In that study, PBT-1 was the most effective of the four PBT analogs investigated. In other studies, we showed that PBT-3 is a potent antagonist of the thromboxane receptor (13) leading to inhibition of collagen aggregation (14) and the second phase of ADP aggregation (15) known to be thromboxane receptor-dependent. Another study showed that PBT-3 is anti-thrombotic in whole blood (16). We recently showed that the PBTs cause K-562 leukemic cells to undergo apoptosis, similar to Gleevec (17). Further, PBT-3 causes the release of cytochrome c from the mitochondria and activates the degradation of caspase 3 (17). In this study, we report the *in vivo* effects of PBT-3 and Gleevec in controlling growth of tumours in the K-562-transplanted mouse model.

Materials and Methods

Cell culture. The CML leukemia cell line K-562 was obtained from ATCC (Manassas, VA, USA). Cells were cultured as described previously (17). Cells were maintained in RPMI 1640 (HyClone) medium supplemented with 10% fetal calf serum (FCS), 100 µg/ml of antibiotics (penicillin and streptomycin) at 37°C in 5% CO₂ at 37°C. Cell viability was assessed with Trypan Blue dye exclusion (>95%).

Animals. Six-week-old female mice (CrI:NU/NU-nuBr) were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). The animals were housed in the Hospital for Sick Children animal facility with free access to chow and water. Animals were injected subcutaneously in the left flank with 1x10⁷ cells/animal and the cells were allowed to grow into solid tumours over the next 2 weeks. Tumour volume was measured with calipers using the formula: V = S² (mm) x L (mm), where S and L are the shortest and longest diameters of the tumour, respectively (18-20). When the tumours reached a volume of 100 mm³, the animals were assigned randomly to control or treatment groups. The groups were treated with either vehicle (100 µl saline containing 7% ethanol),

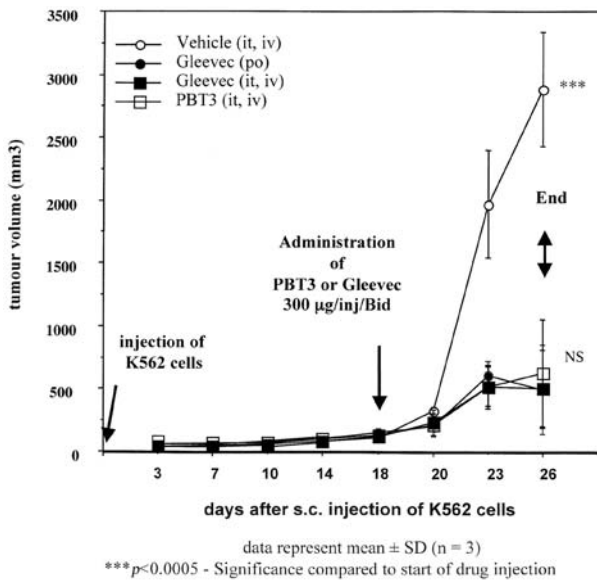


Figure 1. Rate of K-562 tumour growth in nude mice treated with vehicle (saline + 7% ethanol), PBT-3 in vehicle or Gleevec in vehicle. Gleevec administration was carried out in two groups of mice, one received the drug orally, the other twice daily via the intravenous and intra-tumour route. PBT-3 was administered daily via the intravenous and intra-tumour route; so was the vehicle-treated group. Treatment was for 8 days. Tumour volume was measured periodically via the method described in the Methods section.

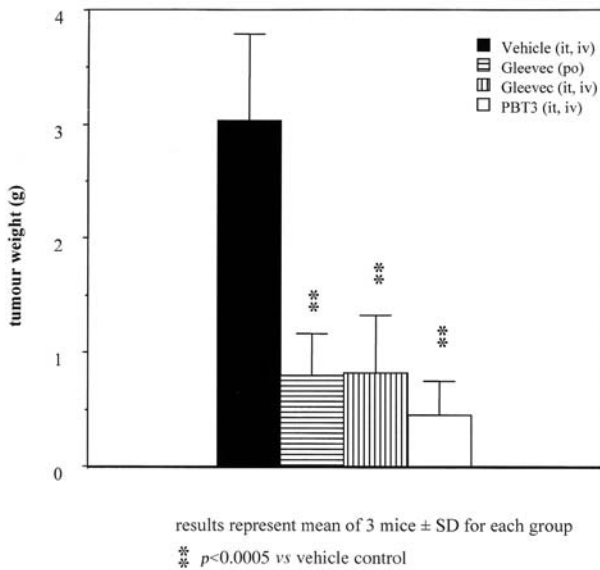
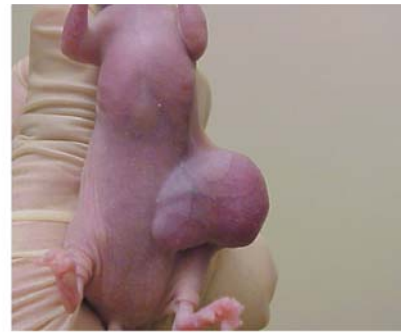


Figure 2. Tumour weights in the various groups measured at the end of the 8-day study.

PBT-3 (300 µg in 100 µl vehicle) or Gleevec (300 µg in 100 µl vehicle). All compounds were administered twice daily, i.e. intra-tumour (morning) and intravenous (afternoon). Another group was administered with Gleevec orally.



Vehicle - treated control



PBT-3 - treated



Gleevec - treated

Figure 3. Photographs taken of a representative animal from each of the groups at the end of the 8th day of treatment after leukemic cell implantation s.c. in nude mice showing tumour growth inhibition by PBT-3 and Gleevec.

Experimental end point. Tumour measurements were taken every 2 or 3 days. Experiments were terminated after 8 days of administration with the drugs (or vehicle) or when tumour volume reached a size greater than 2500 mm³. The relative tumour volume (growth rate) was calculated according to: tumour growth rate = $(V_1 - V_0) / V_0 \times 100\%$, where V_1 is final volume and V_0 is initial volume.

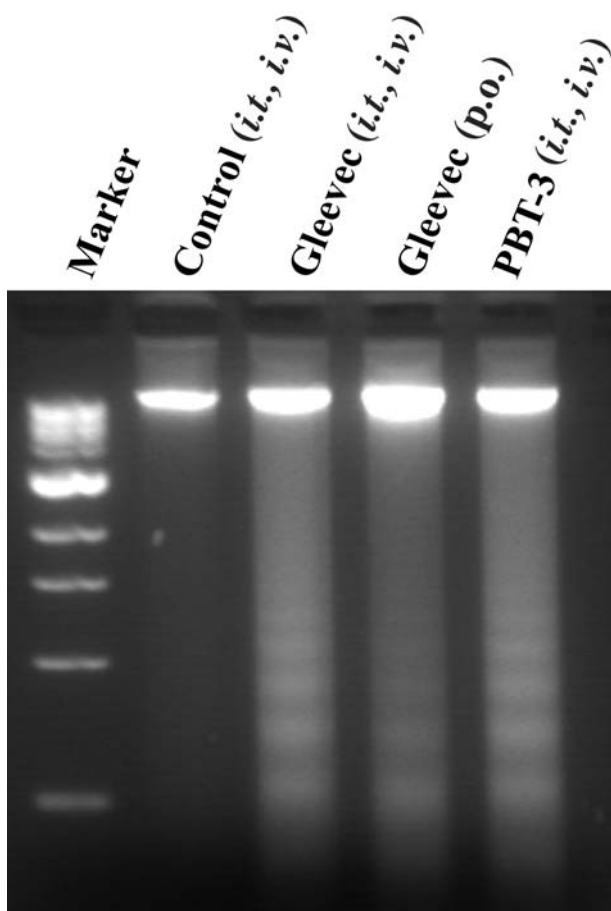
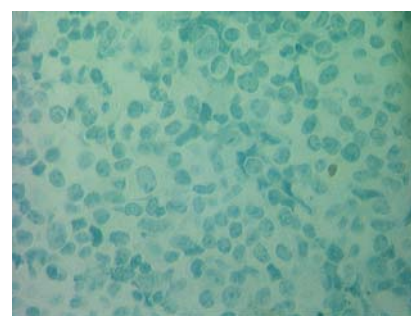


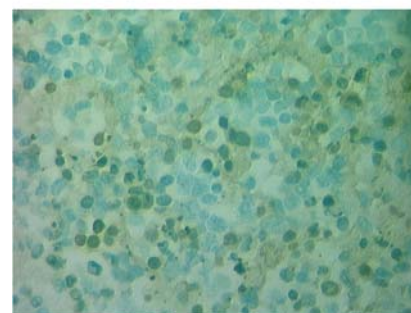
Figure 4. DNA gel electrophoresis showing DNA laddering in tumours from the Gleevec and PBT-3 groups as an indication of *in vivo* apoptosis.

DNA fragmentation assay. For determination of apoptotic death, the pattern of DNA was assessed by DNA electrophoresis (21-23). The tumour DNA in frozen tumours from tumour-bearing mice was extracted with DNeasy tissue kit (Qiagen). The DNA samples were loaded on 2% agarose gel containing ethidium bromide. DNA fragments of known size were used as a reference marker.

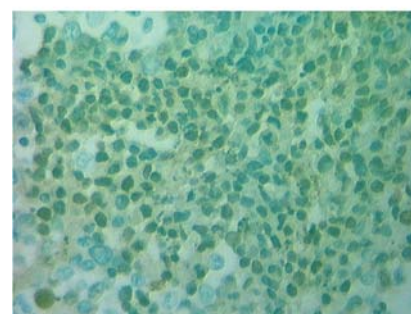
TUNEL assay. Tumour tissue from the PBT-3 group was fixed in 4% paraformaldehyde-PBS at 4°C overnight. The fixed tissue was passed through an ethanol series and embedded in paraffin. The embedded tumour tissue was serially sectioned at 5 µm and placed on poly-L-lysine-coated glass slides. The sections were dewaxed and dehydrated by transfer through an ethanol series. The sectioned tumour tissues on slides were further treated by the TUNEL method using an ApopTag kit according to the manufacturer's instructions (Oncor, USA) (24, 25). The specimens on slides were incubated at 37°C for 2 h in TdT (terminal deoxynucleotide transferase) enzyme to catalyse digoxigenin – dUTP and dATP to 3' ends of fragmented DNA to form a heteropolymer of digoxigenin –11- dUTP and dATP, followed by anti-digoxigenin-peroxidase antibody for 1 h at room temperature. The specimens were visualized by addition of DAB (dimethylaminoazobenzene) and photographed under a light microscope.



Vehicle-treated



PBT-treated



Gleevec-treated

Figure 5. TUNEL assay measuring DNA fragmentation in tumours from the various groups indicating significant apoptosis in the PBT-3 and Gleevec groups.

Results

Figure 1 shows the progress of tumour growth during treatment of groups of mice with the compounds or vehicle. Control tumours grew rapidly, while the growth of tumours were inhibited in the PBT-3 and Gleevec groups ($p < 0.0005$ vs control). In this study, PBT-3 caused a slower growth than Gleevec administered either orally or intravenously/intra-tumour. All animals showed normal behaviour with respect to weight gain, movement and

excitability to the compounds under study. Typical photographs of the animals from each of the groups are shown in Figure 2 at the end of the 8-day study. Tumour weights at the end of the study are shown in Figure 3; control 3.1 ± 0.7 , Gleevec (oral) 0.7 ± 0.4 ($p < 0.0005$ vs control), Gleevec (i.t./i.v.) 0.7 ± 0.5 ($p < 0.0005$ vs control), PBT-3 0.5 ± 0.3 ($p < 0.0005$ vs control).

To determine whether the tumours in the PBT-3- and Gleevec- treated animals were growth inhibited through apoptosis, we subjected the isolated tumours to DNA laddering and to the TUNEL assay. Figure 4 shows significant DNA laddering in the PBT-3 and Gleevec groups but not in the vehicle group. TUNEL assay confirmed the fragmentation of DNA in the growth-inhibited tumours for these animals (Figure 5).

Discussion

Previous studies with the PBT analogs of the hepxilins have shown that these compounds arrest the growth of K-562 cells *in vitro* through a mechanism involving the cytochrome c/caspase 3 pathway (17). PBT-3 was shown to act earlier than Gleevec; apoptosis was observed with PBT-3 at 1-2 h after treatment, with Gleevec >6 h after treatment of the cells. Both PBT-3 and Gleevec caused the release of cytochrome c from the mitochondria and degradation of caspase 3 to the 17 kDa fragment. The question that we needed to address was whether PBT-3 would be effective *in vivo* in arresting the growth of tumours derived from the transplantation of K-562 cell in NU/NU mice. We have shown earlier that PBTs are effective *in vivo* in preventing the onset of lung fibrosis in mice evoked by the chemotherapeutic drug, bleomycin (12). Hence, the compounds are bioavailable and appear to have an anti-inflammatory action. In another study, we have shown that PBT-3 antagonizes platelet aggregation evoked by collagen or the thromboxane agonists I-BOP, or U46619 (14). PBT-3 also inhibits primary, platelet-related hemostasis in whole blood (16). PBT-3 was shown to be more selective as an antagonist of the TP α isoform of the thromboxane receptor (13). In the present study, we showed that PBT-3 is effective in controlling the growth of K-562 cell-derived tumours in mice *in vivo*. At the dose used, PBT-3 appears to be at least as effective as Gleevec, a well-known drug that is effective against CML. Hence, PBT-3 has anti-thrombotic, anti-inflammatory and pro-apoptotic effects. We feel that our study shows that the PBTs may provide alternate and possibly complementary therapy to Gleevec. Further studies are warranted to investigate whether a combination of PBT-3 and Gleevec would be synergistic, thereby improving outcome with a reduction in the required dosage of Gleevec.

Acknowledgements

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