Hepoxilin Analogs Inhibit Bleomycin-Induced Pulmonary Fibrosis in the Mouse

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ABSTRACT

Bleomycin has been suggested to incite plasma extravasation and influx of inflammatory cells leading to pulmonary fibrosis. We hypothesized that stable analogs of the 12-lipoxygenase product, hepoxilin, may attenuate these effects. We initially investigated the effects of the four hepoxilin analogs (PBT-1 to -4) coadministered intradermally with bleomycin and found that PBT-1 and -2 significantly opposed the vascular permeability effects of bleomycin in rat skin. We subsequently tested the hepoxilin analogs for their actions in opposing the intratracheal bleomycin-evoked acute inflammatory phase of lung fibrosis in the mouse, characterized by a marked accumulation of macrophages and an increase in the rate of collagen synthesis and deposition. We found that the bleomycin-evoked effects on macrophage influx were inhibited by all the hepoxilin analogs (PBT-1, -3, and -4 > PBT-2) administered i.p. for 8 days. Increased total lung collagen was completely abrogated by PBT-1 and -2, whereas PBT-3 and -4 had little effect. A dose-response study with PBT-1 indicated that the effective dose for inhibition of bleomycin-induced inflammatory and histological changes was below 10 μg/day. These studies demonstrate an in vivo action of stable analogs of hepoxilin and support an effect on inflammation and vascular permeability from these novel compounds, especially for PBT-1.

Idiopathic pulmonary fibrosis is a devastating disorder that is poorly understood and resistant to treatment (Cooper, 2000). The observation that the antibiotic bleomycin sulfate (BL), a potent cancer chemotherapeutic agent (Adamson, 1984; Nici et al., 1998), may cause interstitial lung fibrosis in humans (Yagoda et al., 1972) led to the development of animal models in which a single dose of BL administered intratracheally induced changes resembling human idiopathic pulmonary fibrosis histopathologically (Kelley et al., 1980). The acute phase of this response is characterized by a marked accumulation of inflammatory cells and an increase in the rate of collagen synthesis and deposition (Cooper et al., 1988; Gurujeyalakshmi and Giri, 1995; Giri and Hollinger, 1996).

Previous animal studies have demonstrated that BL-evoked lung fibrosis is exacerbated with nordihydroguaiaretic acid, a lipoxygenase inhibitor, suggesting that a lipoxygenase product may be involved in endogenous mechanisms controlling lung fibrosis (Giri and Hollinger, 1996). The hepoxilins (HXs) may be candidates for the control of lung fibrosis as they are formed through the 12-lipoxygenase pathway of metabolism of arachidonic acid (Pace-Asciak et al., 1983; Pace-Asciak, 1984; Pace-Asciak and Martin, 1984). These compounds have previously been shown to have significant biological actions (Pace-Asciak, 1984; Dho et al., 1990; Laneuville et al., 1992; Pace-Asciak et al., 1995; Reynaud et al., 1996, 1999; Sutherland et al., 2000). HXs raise free intracellular calcium in human neutrophils ex vivo through the release from stores and vascular tissue in vitro and block the intracellular calcium rise evoked by inflammatory mediators (Laneuville et al., 1993). HX formation is stimulated by inflammatory mediators in the skin (Wang et al., 1999a,b) and in psoriasis (Anton and Vila, 2000), suggesting that they may be involved in anti-inflammatory mecha-
with bleomycin and saline alone. PBT-1 and -2 when coadministered with bleomycin and saline compared mean and saline injections of BL (12 g in saline) to control (open bar), saline + bleomycin (saline; filled bar), and saline + bleomycin + PBT-1 to -4 (filled bars). Bars represent mean ± S.E.M. (n = 6 per group). *p < 0.05, by one-way ANOVA, for PBT-1 and -2 when coadministered with bleomycin and saline compared with bleomycin and saline alone.

Experiments were undertaken to determine whether stable analogs of HX exhibit anti-inflammatory activities in vivo, first on BL-evoked changes in skin vascular permeability followed by changes in the lung, using the well-established, intracheal, BL-induced pulmonary fibrosis mouse model (Kelley et al., 1980).

**Experimental Procedures**

**Materials.** HX analogs (PBT-1–PBT-4) were prepared as previously described (Demin and Pace-Aciak, 1993). BL, formamide, hematoxylin, collagen type I, eosin, Evans blue, Fast Green FCF, and picric acid-saturated solution were purchased from Sigma-Aldrich (St. Louis, MO); Direct Red 80 was obtained from Aldrich Chemical Co. (Milwaukee, WI). Sirius Red from Pfaltz and Bauer (Waterbury, CT), enflurane from Zeneca Pharma (Mississauga, ON, Canada), and Inactin [ethyl-(1-methylpropyl)malonylthioiu-rea] from BYK Gulden (Constanza, Germany). Rat monoclonal antibody to murine macrophage F4/80 antigen was purchased from BMA Biomedicals (Augst, Switzerland). Goat anti-rat IgG-biotin was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Avidin-biotin-peroxidase complex immunochemistry kits were purchased from Vector Laboratories (Burlingame, CA). Animals were obtained from Charles River Canada (St. Constant, QC, Canada).

**Institutional Review.** All animal procedures were conducted according to criteria of the Canadian Council for Animal Care, and approval was obtained from the Animal Care Review Committee at The Hospital for Sick Children.

**In Vivo Vascular Permeability.** Male Wistar rats (190–230 g) were anesthetized with Inactin (120 mg/kg), and the dorsal area was shaved. The trachea and left jugular vein were cannulated, and 35 mg/kg Evans blue dye was administered i.v. 5 min prior to intradermal injections of BL (12 mg in saline), BL + HX analogs (PBT-1–PBT-4) 100 ng in saline, and HX analogs alone and saline alone. After 5 min, the animals were decapitated, patches of skin containing the injection site were punched out, weighed, and incubated in 3 ml of formamide at 70°C in glass tubes. After 18 h, the tubes were cooled on ice and centrifuged, and the supernatant was removed. The absorbance at 620 nm was read spectrophotometrically (Laneuville et al., 1991). The color intensity was compared between BL alone to BL + HX analogs, saline + HX analogs, or saline alone.

**Lung Injury and Interventions.** Pulmonary fibrosis high-responder male CBA/J mice (28–30 g, 10 weeks old) were maintained under a controlled environment (temperature 24 ± 1°C, relative humidity 50–55%, 12-h light cycle). A total of 70 mice were studied. Under inhaled anesthesia with enflurane, animals received BL intratracheally (0.2 U in 50 μl of saline) or saline only (controls). The BL group additionally received either saline or each of the four HX analogs (PBT-1, 10, 50, or 100 μg; and PBT-2–PBT-4, 100 μg each) in saline (100 μl) i.p. 1 day before BL administration and each day thereafter until sacrifice. Animals were sacrificed 7 days after BL administration.

**Histology and Immunohistochemistry.** Animals were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (20 mg/kg). The pulmonary circulation was flushed with phosphate-buffered saline containing 1 U/ml heparin to clear the lungs of blood, and perfusion was fixed with 4% (w/v) paraformaldehyde and 0.2% glutaraldehyde while a constant airway pressure of 10 cm of H2O was maintained via a tracheal catheter. The lungs were embedded in paraffin and cut in 5-μm sections. Sections were mounted on α-aminopropyl-triethoxysilane-coated slides. Random sections from all animals in each group were stained with H&E. Collagen in the lung sections was stained with Sirius Red F3B (0.1%) and Fast Green FCF (0.1%) in a saturated aqueous solution of picric acid (Gascon-Barre et al., 1989; Nagler et al., 1999). Alveolar and interstitial pulmonary macrophages were identified and counted by immunohistochemistry on paraffin-embedded sections using an avidin-biotin-peroxidase complex method (Hsu et al., 1981) and then counterstained with Carazzi hematoxylin, dehydrated, cleared in xylene, and mounted. Dilutions of the primary and secondary antibodies were 1:50 (4 μg/ml) and 1:250, respectively. Antibody specificity was verified by omitting the primary antiserum. Macrophages were quantified by counting positively stained cells per random high-power microscopic field (four animals per group and average of four fields per animal).

**Quantification of Lung Collagen.** Collagen content in lung tissue was quantitated by a sensitive spectrophotometric method (Marotta and Martino, 1985) utilizing precipitation of collagen with Sirus Red in acetic acid solution. Absorbance values of the dye solution after precipitation with collagen decrease linearly with increasing collagen concentration. After sacrifice, the thoracic contents were removed en bloc. The right lung was separated from the heart, airways, and major pulmonary vessels and homogenized in 0.5 M acetic acid. The homogenate was sonicated at 40 W for 30 s, left on ice for 30 min, and then centrifuged at 1000g for 10 min to remove insoluble debris. Samples (20 μl in 1 ml of 0.5 M acetic acid) and collagen type I standards (0–500 μg/ml in 0.5 M acetic acid) were added to an equal volume of Sirius Red (120 μg/ml) in 0.5 M acetic acid. The mixture was left at room temperature for 30 min and then centrifuged at 3000 rpm for 10 min. Absorbance of samples was measured at 540 nm and plotted against standards with known concentration of collagen type I.

**Analysis of Data.** Values are expressed as the mean ± S.E.M. Statistical significance (p < 0.05) was determined using Student’s t test or one-way ANOVA with assessment of differences using Duncan’s multiple range test (Snedecor and Cochran, 1980).

**Results**

Evans blue dye leakage, as a marker of vascular permeability, was quantified and compared between saline alone,
BL in saline, and BL + HX analogs (PBT-1–PBT-4) in saline, as shown in Fig. 1. A significant inhibition \((p < 0.05)\) of the BL-evoked plasma leakage was observed with PBT-1 and PBT-2, which were comparable to saline alone, whereas PBT-3 and PBT-4 had no effect relative to BL alone \((p > 0.05)\).

The histopathological changes from representative mice lung sections stained with H&E are shown in Fig. 2. The lung sections from BL-exposed animals showed severe lesions (Fig. 2B), including diffuse alveolar hemorrhage, edema, and cellular infiltrate in the alveolar space and interstitium, compared with saline-exposed controls (Fig. 2A). Treatment with PBT-1 (Fig. 2, C, D, and E) and PBT-3 (Fig. 2G) markedly attenuated the BL-induced changes. The BL effects were only partially improved in the lungs from groups treated with the other HX analogs (Fig. 2, F and H).

Lung sections stained with Sirius Red for collagen are shown in Fig. 3. The sections from the BL-exposed, saline-treated group (Fig. 3B) showed a marked increase in collagen deposition around vessels and airways (stained red) compared with saline-exposed controls (Fig. 3A). Similar to the improvements seen on H&E staining, treatment with PBT-1 (Fig. 3, C, D, and E) significantly opposed the BL effect, resulting in markedly reduced collagen content. The attenuation of BL-induced collagen deposition increased with increasing dose of PBT-1 (Fig. 3, C, D, and E). PBT-2 (Fig. 3F) and PBT-3 (Fig. 3G) showed some inhibitory effects on BL-induced collagen deposition in the lungs, whereas PBT-4 (Fig. 3H) showed little effect.

Collagen content in the right lungs of animals was greatly increased by BL exposure, compared with saline-exposed controls \((p < 0.05;\) Fig. 4). PBT-1 and -2 significantly \((p <

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**Fig. 2.** H&E staining of lung sections. Mice received either i.t. saline (control) or 0.2 U of BL. The BL-exposed mice additionally received either i.p. saline (control and BL alone) or PBT-1 to -4 (BL+) on the day prior to i.t. injection and daily thereafter for 7 days. Bar length = 625 \(\mu\)m. Insets, high-power view of areas enclosed by boxes. A, saline-exposed control animals. B, BL-exposed animals treated with saline showed severe changes, including diffuse alveolar hemorrhage, edema, and cellular infiltrate in the alveolar space and interstitium compared with controls. C, D, and E, BL-exposed animals treated with PBT-1 showed a marked reduction in BL-induced changes at all doses tested. F. BL-exposed animals treated with PBT-2 showed partial improvement in BL-induced changes. G. BL-exposed animals treated with PBT-3 had reduction in BL-induced changes similar to PBT-1-treated animals. H. BL-exposed animals treated with PBT-4 showed partial improvement in BL-induced changes.
0.05) reduced total collagen compared with BL-exposed, saline-treated controls. PBT-3 and -4 were not effective in preventing lung collagen deposition, which is significantly increased relative to non-BL-exposed, saline-treated animals \((p < 0.05)\).

Alveolar and interstitial macrophage content in the lung was greatly increased by BL exposure, compared with saline-exposed controls (Fig. 5). All the HX analogs significantly \((p < 0.05)\) reduced the macrophage number compared with BL-exposed, saline-treated controls. PBT-1 was also efficacious at lower doses of 10 and 50 \(\mu g/day\) \((p < 0.05)\) compared with BL-exposed saline-treated animals; Fig. 5). PBT-2 was not as effective as the other HX analogs in reducing macrophage number, which are significantly increased relative to non-BL-exposed, saline-treated animals \((p < 0.05)\).

**Discussion**

Bleomycin, in a single dose administered intratracheally, induces highly reproducible inflammatory and fibrotic pulmonary changes in mice (Cooper et al., 1988), which has been useful in directing research toward treatment strategies for pulmonary fibrosis in humans (Cooper, 2000). The early lesions include diffuse alveolar hemorrhage and marked accumulation of numerous lymphocytes, plasma cells, and macrophages. Subsequently, increased numbers of fibroblasts secrete a collagenous extracellular matrix that progressively thickens the alveolar interstitium and severely compromises pulmonary function.

The mechanisms of HX protection against lung fibrosis may include the following. First, previous studies have dem-
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Asciak et al., 1995), we prepared chemically stable analogs in which the unstable epoxide is replaced by a stable cyclopropyl group (Demin and Pace-Asciak, 1993). These compounds mimic the actions of the native products (C. Pace-Asciak, unpublished observations). Hence, we speculated that the HX analogs might have anti-inflammatory actions in vivo.

Our results demonstrated that the HX analog, PBT-1, eliminated BL-induced alveolar hemorrhage, macrophage infiltration, and collagen deposition. The inhibitory effects of PBT-1 were evident at a dose as little as 10 μg/day i.p. PBT-2, but not PBT-3 or -4, was also a potent inhibitor of collagen deposition when measured in the total lung. Of note, we found that the effects of PBT-2 on macrophage accumulation were reduced in comparison to its high potency against BL-induced vascular permeability in the skin and lung collagen deposition. Since pulmonary collagen deposition induced by BL has been suggested to involve plasma extravasation (Thrall et al., 1979), we believe that PBT-1 and -2, may have inhibited collagen deposition by attenuating BL effects on vascular permeability. This may also explain the lack of efficacy of PBT-3 and -4, which we found to be ineffective in preventing BL-induced increased vascular permeability in the skin. Our finding that all of the HX analogs were effective (PBT-2 less than the others) inhibitors of macrophage influx indicate that macrophages may not be directly related to increased vascular permeability and collagen deposition induced by BL. The precise mechanisms by which the HX analogs exert their effects in vivo and an understanding of structure-activity relationships of the different analogs require further elucidation. We conclude that these novel compounds warrant further investigation as potential therapeutic agents for disorders involving increased vascular permeability, inflammatory cell influx, and fibrosis.

References

Fig. 4. Effects of HX analogs on BL-mediated increased right lung collagen content. Mice received either i.t. saline (open bars) or 0.2 U of bleomycin (filled bars) and either i.p. saline (control) or PBT-1 to -4 (100 μg) on the day prior to i.t. injection and daily thereafter for 7 days. Bars represent mean ± S.E.M. (n = 3–5 per group). *, p < 0.05, by one-way ANOVA, for BL-exposed, saline-, PBT-3-, and PBT-4-treated animals compared with non-BL-exposed animals. #, p < 0.05, by one-way ANOVA, for BL-exposed, PBT-1-, and -2-treated compared with BL-exposed saline-treated animals.

Fig. 5. Effects of HX analogs on BL-mediated increased lung macrophage content. Mice received either i.t. saline (open bar) or 0.2 U of bleomycin (filled bars) and either i.p. saline (control), PBT-1 (10, 50, or 100 μg), or PBT-2 to -4 (100 μg) on the day prior to i.t. injection and daily thereafter for 7 days. Bars represent mean ± S.E.M. (n = 4 per group). *, p < 0.05, by one-way ANOVA, for BL-exposed, saline-treated animals compared with all other groups. #, p < 0.05, by one-way ANOVA, for BL-exposed PBT-2-treated compared with control animals.

onstrated an exacerbation of the BL-evoked lung fibrosis by nordihydroguaiaretic acid (Giri and Hollinger, 1996), a lipoxygenase inhibitor, suggesting that some lipoxygenase product (possibly HX) may be involved in lung protection. Second, native HXs block the calcium-mobilizing actions (release from intracellular stores) evoked by inflammatory mediators as diverse as leukotriene B4, platelet activating factor, and f-met-leu-phe (Laneuville et al., 1993). Third, HXs possess actions on vascular permeability (Laneuville et al., 1991), a process that is believed to accompany the actions of BL in causing lung fibrosis (Thrall et al., 1979; Adamson, 1984). Because the native HXs are rather unstable chemically and biologically, due to the presence of an allylic epoxide (Demin et al., 1990) and epoxide hydrolases in cells (Pace-Asciak et al., 1995), we prepared chemically stable analogs in which the unstable epoxide is replaced by a stable cyclopropyl group (Demin and Pace-Asciak, 1993). These compounds mimic the actions of the native products (C. Pace-Asciak, unpublished observations). Hence, we speculated that the HX analogs might have anti-inflammatory actions in vivo.


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