Mutat Res Gen Tox En 829–830 (2018) 19–25

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

journal homepage: www.elsevier.com/locate/gentox

Genotoxicity assessment of the novel antitussive agent Benzonatate and its major metabolite

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ARTICLE INFO

Keywords:
Benzonatate
Ames
Chromosomal aberration
Mouse micronucleus
Carcinogenicity

ABSTRACT

Benzonatate (TESSALON®) is a peripherally acting oral antitussive. It undergoes rapid ester hydrolysis producing 4-(butylamino) benzoic acid (BBA) and methylated polyethylene glycol (MPG) metabolites, which are eliminated in urine and feces. The nonclinical and clinical efficacy of Benzonatate has been demonstrated over the last 60 years, but its safety was not fully assessed. In this study, we tested the genotoxicity of Benzonatate and its major metabolite BBA in an in vitro bacterial reverse mutation and in vivo micronucleus assays. A chromosomal aberration assay was also performed on Benzonatate and BBA. In the reverse mutation assay, Benzonatate and BBA doses 1.5–5000 µg/plate ± S9 metabolic activation were used and the numbers of revertants/plate were compared to various controls. Chromosomal aberration assays with human peripheral blood lymphocytes used Benzonatate and BBA concentrations 25–2000 and 62.5–1930 µg/mL, respectively. A CByB6F1 mouse bone marrow micronucleus assay was performed as part of a 28-day oral toxicology study at up to 250 mg/kg/day. The frequencies of micronuclei in polychromatic erythrocytes in treated groups were compared with the control group. Neither Benzonatate nor BBA induced significant mutagenicity in any of the bacterial strains, with or without metabolic activation. They also did not produce any biologically relevant structural or numerical aberrations in human chromosomes. Benzonatate and its BBA and MPG metabolites rapidly produced from esterase activity did not produce any significant increase in the incidence of micronucleated polychromatic erythrocytes. In conclusion, Benzonatate and its major metabolite BBA were not mutagenic and did not cause numerical or structural chromosome alterations. While the MPG metabolite was not tested, studies on structural analogues indicated it was also unlikely to be genotoxic. This was supported by oral rodent carcinogenicity assays showing no increase in malignancies.

1. Introduction

Benzonatate (CAS # 104-31-4; TESSALON®) is a non-narcotic, peripherally acting, oral prescription antitussive indicated for the symptomatic relief of cough. It was approved in the United States in 1958. The recommended dosage is 100 or 200 mg 3 times a day in adults and children over 10 years of age [1]. Benzonatate consists of a mixture of 15 structural analogues/oligomers that differ in the number of the repeating side chain $(OCH_2CH_2)_n$, where n = 7–50. Its chemical structure is related to the ester class local anesthetic agents such as procaine and tetracaine (Fig. 1). Benzonatate acts peripherally through its anesthetic properties, dampening the activity of stretch receptors located in respiratory passages, lungs and pleura, which mediate the cough reflex [2–4]. It has no inhibitory action in the respiratory center. As with the local anesthetics, Benzonatate is susceptible to ester hydrolysis, producing the *N*-butyl substituted *para*-aminobenzoic acid (PABA) metabolite, 4-(butylamino) benzoic acid (BBA) (CAS # 4740-24-3) and methylated polyethylene glycol (MPG) [5].

Benzonatate's efficacy has been demonstrated over the years. To add to the nonclinical safety database, a standard genotoxicity battery was performed on Benzonatate and BBA. This consisted of a bacterial reverse mutation assay (Ames test), chromosomal aberrations assay in human peripheral blood lymphocytes (HPBL) and a bone marrow micronucleus assay performed as part of a 28-day Benzonatate toxicology study in CByB6F1 mice. All studies were performed at MilliporeSigma BioReliance® Toxicology Services (Rockville, MD) under Good

Abbreviations: BBA, 4-(butylamino) benzoic acid; CP, cyclophosphamide; DMSO, dimethyl sulfoxide; HPBL, human peripheral blood lymphocytes; ICH, International Conference on Harmonization; MPCE, micronucleated polychromatic erythrocyte; MPG, methylated polyethylene glycol; PABA, *para*-amino benzoic acid; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocyte

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https://doi.org/10.1016/j.mrgentox.2018.03.007
Received 13 December 2017; Received in revised form 20 March 2018; Accepted 21 March 2018
Available online 21 March 2018

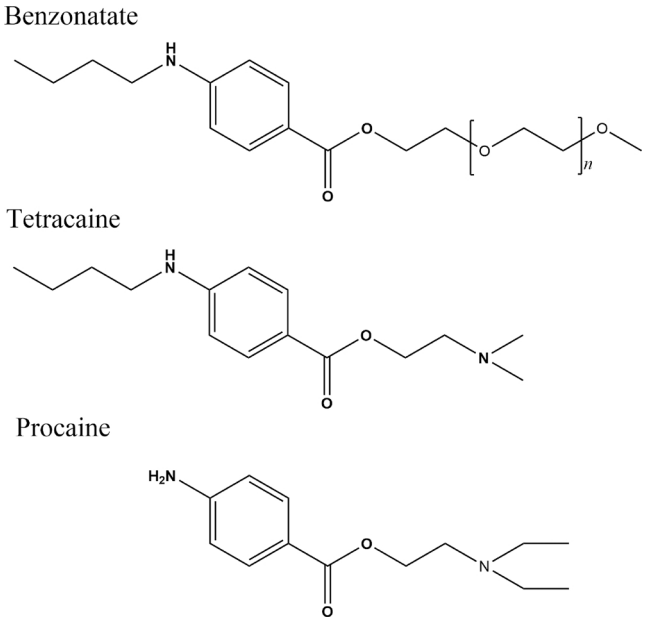

Fig. 1. Structures of Benzonatate and its analogues.

Laboratory Practice guidelines [6].

2. Materials and methods

2.1. Chemicals and reagents

Benzonatate (CAS number 104-31-4; 2, 5, 8, 11, 14, 17, 20, 23, 26-nonaoxaoctacosan-28-yl *p*-(butyl amino) benzoate; purity 99.6%) is a light yellow colorless liquid (BASF, Florham Park, NJ). 4-(Butylamino) benzoic acid (BBA; CAS number 4740-24-3; purity 99.8%) is an off-white powder (Sigma-Aldrich, St Louis, MO). They were both stored at room temperature under nitrogen, protected from light. Sterile distilled deionized water (Gibco, Carlsbad, CA) was used as the vehicle. Positive controls 2-nitrofluorene, sodium azide, 9-aminoacridine, methyl methanesulfonate, 2-aminoanthracene, dimethylbenz[*a*]anthracene, cyclophosphamide (CP), mitomycin, and trifluorothymidine were obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was obtained from EMD Chemicals (Billerica, MA). Deionized water was used as the vehicle for Benzonatate and sodium azide, and DMSO for BBA and positive controls. Aroclor 1254-induced rat liver S9 was used as the metabolic activation system and was obtained from MolTox (Boone, NC).

2.2. Bacterial reverse mutation assay (Ames)

The assay was performed in 2010 according to the testing guidelines (current at that time) of the International Conference on Harmonization (ICH) [7,8] and Organization of Economic Cooperation and Development (OECD) [9]. The tester strains used were *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535, and TA1537, and *E. coli* strain WP2 *uvr*A, in the presence and absence of a rat liver metabolic system (S9). *S. typhimurium* tester strains were obtained from Dr. Bruce Ames' master cultures and the *E. coli* tester strain was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland); both species of tester strain were distributed by MolTox (Boone, NC). Tester strains TA98 and TA1537 are sensitive to frameshift mutagens which can revert their histidine dependence (auxotrophy) to histidine independence (prototrophy). Reversions occur in tester strain TA100 in response to mutagens that cause both frameshift and basepair substitution mutations. Tester strains TA1535 and *E. coli* undergo reversions in response to base-pair-substituting mutagens.

The study used the plate incorporation technique described by Ames [10] and updated [11]. Plates were incubated for 48–72 h at 37 ± 2 °C. The condition of the bacterial background lawn was evaluated for evidence of test articles toxicity by a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plates. Revertant colonies for a given tester strain and activation condition were counted either by automated colony counter or by hand, unless the plate exhibited toxicity.

Benzonatate and BBA solutions were prepared immediately before use. An initial toxicity-mutation assay was performed to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary evaluation of the mutagenicity. Vehicle control (deionized water), positive controls and a minimum of eight doses of both test articles were plated with overnight cultures of *S. typhimurium* and *E. coli* tester strains on selective minimal agar ± S9. Doses tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 μg/plate. No mutagenic responses were observed ± S9. Since no precipitate or background lawn toxicity was observed, the maximum dose in the confirmatory mutagenicity assay was 5000 μg/plate. The positive controls, vehicle controls as well as at least five doses of the test articles were plated in triplicate with overnight cultures of *S. typhimurium* and *E. coli* tester strains on selective minimal agar ± S9. The doses tested were 15, 50, 150, 500, 1500 and 5000 μg/plate for Benzonatate and 50, 150, 500, 1500 and 5000 μg/plate for BBA, and these met the OECD 471 guideline requirements (≥ five doses up to 5000 μg per plate with ≥ three non-toxic levels).

2.3. In vitro mammalian chromosome aberration assay

The assay was performed in 2010 according to the testing guidelines (current at that time) of the ICH [7,8] and OECD [12]. Due to issues on the availability of Human Peripheral Blood Lymphocytes (HPBL), lymphocytes were obtained from a healthy non-smoking 24-year old male and 31-year old female for the Benzonatate and BBA assays, respectively. Results from the two donors were not pooled. Control results for both donors were similar and within the historical control range. The metabolic S9 system was mixed with a cofactor pool of 2 mM $MgCl_2$, 6 mM KCl, 1 mM glucose-6-phosphate, 1 mM NADP, and 20 μL S9/mL medium (RPMI 1640 serum-free medium supplemented with 100 units penicillin/mL, 100 μg streptomycin/mL, and 2 mM L-glutamine).

A preliminary toxicity assay was performed to select assay concentrations and evaluate test articles' effects on mitotic index. Cells were exposed to the vehicle control (deionized water) and 0.5, 1.5, 5, 15, 50, 150, 500, 1500, 5000 μg/mL test articles for 4 h ± S9 and 20 h continuously −S9. Approximately 0.6 mL of heparinized blood was inoculated into centrifuge tubes containing 9.4 mL of RPMI-1640 complete medium (RPMI-1640 containing 15% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/mL, 100 μg streptomycin/mL) supplemented with 1% phytohemagglutinin. The tubes were incubated at 37 ± 1 °C at 5 ± 1% CO_2 for 44–48 h 2 h prior to the scheduled cell harvest, 0.1 μg/mL Colcemid® was added to the cultures and returned to the incubator until cell collection. Cells were collected by centrifugation, treated with 0.075 M KCl, fixed, stained and the number of cells in mitosis/500 cells per concentration scored to determine any effect on mitotic index.

The subsequent chromosome aberration assay was performed by exposing duplicate cultures of HPBL to 25, 50, 150, 500, 1000 and 2000 μg/mL test articles and controls as described above. Selection of doses was based upon precipitation of the test article in treatment medium or cytotoxicity. In the presence of precipitation, the highest dose concentration was the lowest precipitating dose regardless of toxicity. In the absence of precipitation, the highest dose was that which induced at least 50% toxicity as measured by mitotic inhibition, relative to vehicle control. Two additional lower dose concentrations