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Genotoxicity of two new carbazole derivatives with antifungal activity

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ABSTRACT

The class of carbazoles includes compounds with high biological activities and broad spectra of action. PLX01107 and PLX01008 are xenomycins, a new subclass of antimicrobial carbazole derivatives demonstrating strong antifungal activity *in vitro*. We performed three tests, a bacterial reverse mutation assay (Ames test), *in vitro* cytokinesis-block micronucleus assay, and chromosome aberration test in mouse bone marrow cells, to investigate the possible genotoxicity of these compounds. Despite their structural similarity, the two compounds had different genotoxicity profiles. PLX01008 showed positive effects in all assays. PLX01107 showed no mutagenicity in the Ames test but demonstrated strong cytogenetic activity *in vitro* and *in vivo*. PLX01107 was also tested in the *in vivo* alkaline comet assay, where a weak but statistically significant increase in DNA damage was seen in liver cells 24 h after treatment. Significantly increased levels of formamidopyrimidine DNA glycosylase (FPG)-sensitive sites were found in bone marrow cells of PLX01107-treated mice (FPG-modified comet assay), suggesting induction of oxidative or alkylation damage to DNA.

1. Introduction

Fungi are a ubiquitous group of eukaryotic organisms consisting of approximately 5 million species [1]. Some species parasitize plants and animals and are causative agents of human diseases [2]. Structural classes of modern antifungal drugs are limited to polyene, azole, allylamine, and echinocandin derivatives [3]. The high prevalence of opportunistic invasive fungal infections in humans and their common resistance to therapy stimulates searches for novel antifungal agents [4]. Carbazole derivatives show a wide spectrum of biological activities, including neuroprotective [5], antiviral [6], antitumor [7], antiprotozoal [8], and antibacterial [9] properties. As part of a drug discovery initiative, Cleveland BioLabs, Inc. synthesized a new class of carbazole derivatives – a 2nd generation of curaxins, compounds with anticancer and antimalarial potential [10]. Some of the curaxins demonstrated anti-infective properties and were assigned to a subclass with the proprietary name "xenomycins". CBL0100 was considered as the most promising compound of this group and was evaluated as a drug candidate; it showed strong antifungal efficacy both *in vitro* and *in vivo*, exhibited no genotoxicity, but failed due to general toxicity [11–13]. CBL0100 was optimized in order to obtain compounds with similar pharmacodynamics profiles but fewer side effects. Two novel xenomycins, PLX01107 and PLX01008, with marked antifungal activity, were designed and synthesized.

The potential genotoxicity of carbazole derivatives is of concern. Genotoxic effects were demonstrated for carbazole itself and some of its derivatives [14–16]. Curaxins do not induce DNA damage, as demonstrated by the comet assay and γH2AX staining *in vitro*, but they are able to intercalate into DNA [10]. In the present study, a bacterial reverse mutation assay (Ames test), an *in vitro* cytokinesis-block micronucleus assay, and a chromosome aberration test in mouse bone marrow cells were performed, to investigate the possible genotoxicity of PLX01107 and PLX01008. PLX01107 was also tested *in vivo* in both the standard and the FPG-modified alkaline comet assay.

2. Materials and methods

2.1. Test substances

PLX01107 and PLX01008 (purity >99% by HPLC) were synthesized by BioBlocks (San Diego, CA, USA). Both compounds were

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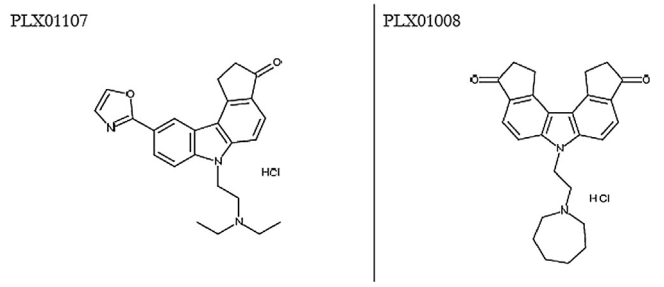

Fig. 1. Structures of xenomycins PLX01107 and PLX01008.

characterized by HPLC, elemental analysis, heavy metals analysis, and melting point. The structures of both compounds were confirmed by ^{1}H NMR, infrared and UV spectroscopy, and LC–MS (m/z 387.48 and 401.2 for PLX01107 and PLX01008, respectively, as free bases). The structures of the compounds are shown in Fig. 1.

2.2. Chemicals

The Ames MPF^{TM} PENTA I kit and Aroclor 1254-induced S9 were purchased from Xenometrix (Allschwil, Switzerland). Phytohemagglutinin (cat.# L1668), RPMI-1640 medium (cat.# R8758), DMSO (cat.# D8418), KCl (cat.# P9541), cytochalasin B (cat.# C6762), methyl methanesulfonate (cat.# 129925) and cyclophosphamide (cat.# 93813) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) (cat.# SH30071.01) was procured from HyClone (South Logan, UT, USA), SYBR Green I (cat.# S7567) from Invitrogen (Carlsbad, CA, USA), and formamidopyrimidine DNA glycosylase (FPG) (cat.# M0240S) from New England BioLabs (Ipswich, MA, USA). Normal-melting agarose (NMA), low-melting agarose (LMA), Triton-X 100, EDTA, and PBS were purchased from Panreac (Barcelona, Spain).

2.3. Animals

All study protocols were approved by the Animal Ethics Committee of the V.V. Zakusov Research Institute of Pharmacology. Experiments were performed on male and female CD-1 mice (20–22 g) procured from the Central Animal Facility of the V.V. Zakusov Research Institute of Pharmacology. The animals were housed (10–12 per cage; conventional polycarbonate cages were used, 1290D, Techniplst, size: 425 × 276 × 153 mm) in the facility with room temperature maintained at 22 ± 2 °C under an automatically controlled cycle of 12 h light:dark. Standard laboratory animal feed (MEST Ltd, Russia) and water were provided ad libitum. Animals were quarantined for at least one week before the beginning of the experiment. During that time, the animals were observed daily for signs of illness. Only healthy animals were used in the study.

2.4. Ethics statement

The study was approved by the Local Bioethics Committee of the V.V. Zakusov Research Institute of Pharmacology. After obtaining written informed consent, healthy non-smoking young male (38 y) and two female (26 and 27 y) individuals were used as blood donors. According to the donors' declarations, they were not exposed to radiation or drug treatment in the recent past.

2.5. Ames microplate format (MPF) test

Experiments were conducted with *S. typhimurium* TA98, TA100, TA1535, TA1537 and combination of *E. coli* WP2 *uvrA* and WP2 [pKM101] bacterial strains using the Ames MPF^{TM} PENTA I kit following the manufacturer's manual [17].

PLX01107 and PLX01008 were dissolved in DMSO. The final concentration of DMSO in the exposure media was 4%. Freshly thawed frozen strains were inoculated in growth medium and the cultures were grown overnight (14–15 h) at 37 °C in an environmental shaker. The test cultures were exposed to the test substances for 90 min in liquid minimal histidine (*S. typhimurium*) or tryptophan (*E. coli*) exposure media. At the end of the exposure period, the cultures received indicator medium, which lacks the required amino acid and selects for prototrophic reversion. The cultures were then distributed to a 384-well microtiter plate and incubated for 48 h. The bromocresol purple in the indicator medium turns yellow as the pH drops, as a result of catabolic activity of revertant bacteria that grow in the absence of the required amino acid. The number of positive (yellow) wells out of 48 wells per replicate and dose was counted by visual inspection.

The assays were performed in triplicate with or without Aroclor 1254-induced rat liver fraction S9. The mean number of positive wells per dose was calculated from the triplicates, and the fold increases above the baseline (mean of negative control plus SD) were determined for each concentration of substance. An increase of more than 2-fold with p-value ≤ 0.05 (Student's t-test) was considered as a positive response. Cytotoxicity was assessed visually by a drop of the number of positive wells to zero and by an increase of the brightness of the purple medium as compared with the vehicle control due to lack or inhibition of bacterial growth.

2.6. The cytokinesis-block micronucleus (CBMN) assay

The CBMN assay was performed as described earlier [18] with slight modifications. Heparinized blood samples were obtained from a healthy (non-smoking) young male (38 y) and two female donors (26 and 27 y). Cultures contained 250 μL whole blood, 375 μL fetal calf serum and RPMI-1640 medium (with L-glutamine) up to 2.5 mL. The final mixture was supplemented with phytohemagglutinin (10 μg/mL) and incubated in 24-well plates at 37 °C.

After 41 h incubation, blood cultures were spiked with test compounds to final concentrations of 2, 10, or 50 ng/mL. PLX01107 and PLX01008 were added to blood cultures as a 10 mM stock in DMSO. The final concentration of DMSO in cultures was 0.1%. Methyl methanesulfonate (40 μg/mL) was used as positive control. Cytochalasin B (Cyt B, 5 μg/mL) was added to the at 44 h of incubation, to arrest cytoplasmic division of the cells in the binucleated state.

In the experiments with S9 metabolic activation, after 3 h incubation with the test compounds, the cell cultures were centrifuged and washed with RPMI medium, 5 mL. After centrifugation, the cell pellet was re-suspended in fresh complete medium and Cyt B was added to those preparations. As a positive control, cells were incubated with cyclophosphamide (20 μg/mL).

Following Cyt B treatment the cultures were incubated for 28 h at 37 °C to achieve a total incubation time of 72 h. Cells were harvested by centrifugation, treated with hypotonic solution (0.55% KCl), and fixed by three rounds of washing with ethanol:glacial acetic acid (3:1 vol/vol). Microscope slides were prepared by dropping the cell suspension on clean slides, air-drying, and staining with 2% Giemsa in phosphate buffer (pH 6.8). All slides were independently coded (blinded) before microscopic examination.

For each culture, micronuclei (MN) were scored in 1000 binucleated cells using the criteria of Fenech et al. [19]. As a parameter for cytotoxicity, the nuclear division index (NDI) was calculated using the formula:

$$NDI = \frac{M1 + 2M2 + 3M3}{N},$$

where M1–M3 is the number of cells with 1, 2 and ≥3 nuclei and N is the total number of cells scored. Each experiment was repeated